Cotton Farming and N Cycling: Adaptation to Climate Change

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STATEMENT OF AUTHENTICATION

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

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LIST OF ABBREVIATIONS

ACRI – Australian Cotton Research Institute

AMO – ammonia monooxygenase

AOB – ammonia-oxidizing bacteria

AOA – ammonia-oxidizing archaea

bp – base pair

C – carbon

C:N – carbon to nitrogen

CO_2 – carbon dioxide

DNA – deoxyribonucleic acid

DAP – day after planting

dwt – dry weight

FACE – free-air CO_2 enrichment

K – potassium

KCl – potassium chloride

MS – the mean sum of squares

N – nitrogen

N_2 – dinitrogen

NH_2OH – hydroxylamine

NO – nitric oxide

N_2O – nitrous oxide

NOB – nitrite-oxidizing bacteria

NH_4^+ – ammonium

NH_3 – ammonia

NO_3^- – nitrate

NO_2^- – nitrite

Nar – nitrate reductase

Nap – nitrate reductase

Nir – nitrite reductase

Nor – nitric oxide reductase

Nos – nitrous oxide reductase

Nxr – nitrite oxidoreductase

NMR – net N mineralization rate

NNR – net nitrification rate

NSW – New South Wales

O_2 – oxygen

OTUs – operational taxonomic units

P – phosphorus

PCO – principal coordinate analysis

PAR – photosynthetically active radiation

PBS – phosphate-buffered saline

ppm – parts per million

PCR – polymerase chain reaction

PNR – potential nitrification rate

PDR – potential denitrification rate

Post-WL – post-waterlogging

Post-PD – post-prolonged drought

RNA – ribonucleic acid

SE – standard error

SS – the sum of square

TRFLP – terminal restriction fragment length polymorphism

TRF – terminal restriction fragment

RT – room temperature

VPD – vapor pressure deficit

WFPS – water-filled pore space

WL – waterlogging

ABSTRACT

Climate models have projected the atmospheric carbon dioxide (CO_2) concentration will double and a 1.1-6.4^{o}C rise in global average temperature by the end of 21st century. Simultaneously, extreme weather events including flooding and drought have been predicted to increase in frequency and intensity. Such changes are expected to have profound effects on agriculture. Soil nitrogen (N) cycling, particularly nitrification and denitrification plays an important role in the availability of N in soils for plant uptake, and hence changes in these processes due to global change may considerably influence crop productivity. These N processes are microbially driven and little is known about the role of soil microorganisms in regulating the process rates and how they respond to environmental disturbances. Therefore, my study aimed to elucidate the responses of soil nitrification to climate change and extreme weather events, and subsequent consequences for crop yields, by using cotton as a model system. Additionally, the legacy effects of extreme weather events and the impacts of added N-fertilizer on soil N, C processes and microbial communities and the subsequent consequences for crop productivity were examined.

First effects of waterlogging on soil nitrification and nitrifying community in cotton farming were examined. This study was a field-based experiment conducted at the Australian Cotton Research Institute (ACRI) in Narrabri, NSW. Waterlogging events simulated by running furrow irrigation for 120 hours were applied at the early and late flowering stages, respectively. Waterlogging had strong effects on soil moisture, pH, potential nitrification rate (PNR) and soil nitrate (NO_3^-) concentration. The abundance of ammonia-oxidizing bacteria (AOB) decreased approximately 10-fold whereas that of ammonia-oxidizing archaea (AOA) decreased about 2-fold after waterlogging. Shifts in AOB and AOA community structures were also observed after waterlogging. Significant correlations between both AOB and AOA communities and PNR were observed; however, AOB was more strongly correlated to PNR than AOA. Significant linear negative

correlations between soil moisture and ammonia-oxidizing communities and PNR were also obtained. These results indicate that waterlogging impacted on soil physicochemical properties, resulting in changes in ammonia-oxidizing communities and nitrification activity.

In the second chapter, the effects of elevated temperature (+1.1^{o}C) alone and elevated temperature in combination with elevated CO_2 (550 ppm) on soil nitrification and nitrifying communities in cotton farming were investigated using field-based environmentally-controlled chambers. This study was conducted at ACRI in Narrabri, NSW. Elevated temperature did not affect soil PNR and AOB community abundance and structure. The AOA community responded significantly to elevated temperatures by increasing its abundance and shifting their community structure. Combined elevated CO_2 and temperature significantly increased both AOB and AOA abundance, and resulted in shifts in AOB and AOA community structures. Both AOB and AOA communities were significantly correlated with PNR, although AOA exhibited a weaker relationship with PNR than AOB. Effects of climate factors on soil nitrification and nitrifying community depended on the stage of cotton growth since treatment effects were only observed when cotton reached the early flowering stage.

Thirdly, the responses of soil N processes (nitrification, denitrification, and N mineralization), functional microbial communities, crop growth and productivity to different N fertilizer regimes (0, 100, 200 and 300 kg N/ha) after exposure to waterlogging and prolonged-drought were investigated by conducting a glass-house experiment at Western Sydney University (WSU). Prolonged-drought prior to cotton planting established a strong legacy effect on soil N processes, ammonia-oxidizing communities, *nosZ*-containing community, plant growth and productivity. N fertilizer application up to 300 kg N/ha could not counteract the legacy effect of prolonged drought on soils and plants although N supply improved soil fertility. These results suggest that the depleted functional microbial communities may take a long time to recover after drought. Waterlogging prior to planting had a legacy effect on soil NO_3^-

content and the *nosZ*-containing community. The legacy effect of waterlogging on soil NO_3^- was diminished completely by N addition. Despite an increase in *nosZ* gene abundance due to waterlogging before sowing, soil N availability and crop growth and productivity was not impacted. However, N loss from the plant-soil system can be significant if further waterlogging occurs, thereby potentially affecting crop yields.

Finally, the responses of the soil bacterial community and microbial respiration to legacy effects of waterlogging and prolonged-drought, and N fertilizer addition were also examined. This experiment was carried out to examine links between N and C cycling in farming systems. Prolonged-drought prior to planting generated a strong legacy effect on soil bacterial abundance, diversity, and composition, and microbial respiration rates. N fertilizer supply increased soil bacterial abundance and diversity, and altered bacterial community composition. However, N fertilizer application up to 300 kg N/ha could not counteract the legacy effects of prolonged-drought on the soil bacterial community. Additionally, different bacterial phyla responded differently to the legacy effects of prolonged-drought and N supply. In contrast to prolonged-drought, waterlogging did not establish a legacy effect on the soil bacterial community and microbial respiration. This suggests that the soil bacterial community might be resistant to waterlogging or recover completely upon water stress. N fertilizer supply inhibited soil microbial respiration by inhibiting C-degraded enzyme activities. Some weak but significant correlations between soil total bacterial community and microbial respiration were observed; however, addition of N fertilizer weakened these relationships further.

Overall, my study provides novel evidence of soil N cycling responses to climate change and extreme weather events in cotton farming systems. My study is the first to demonstrate a legacy effect from extreme weather events and external N supply on soil N and C processes, and subsequent consequences on crop productivity. Data obtained in this study will support the development of robust predictive models and adaptation strategies to sustain crop yields under future climatic conditions via effective N management.

CHAPTER 1 GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Crop productivity and environmental impacts

Agriculture is a major land use in the world with approximately 1.2-1.5 billion hectares being utilized for cropping systems to secure global food and fiber demand (Howden *et al.*, 2007). It is crucial to increase crop productivity to meet the future requirements of the human population, which is predicted to increase to 9 billion people by the middle of the 21st century (Howden *et al.*, 2007, Barnosky *et al.*, 2012). Increasing crop productivity is dependent on key factors including weather, climate and soil fertility, and thus changes in these environmental conditions are likely to substantially alter potential, future crop yields (Lobell & Gourdji, 2012, Shang *et al.*, 2014).

Crop productivity is strongly affected by climatic variables including temperature, concentrations of atmospheric CO_2, and the year-to-year variability of weather such as solar radiation, humidity and precipitation (Gornall *et al.*, 2010). Temperature is a main factor determining the growth and development of plants while CO_2 directly influences key physiological processes, such as photosynthesis (Haferkamp, 1988, Campbell *et al.*, 1988). Regarding weather, the timing, type and amount of rainfall can affect plant growth (Godwin, 1990). Thus, a prediction of highly variable precipitation into the future is expected to considerably influence crop yields (Lobell & Gourdji, 2012).

Soil plays a key role in maintaining high crop productivity as it contains a pool of organic matter and diverse microorganisms which are drivers of nutrient cycling; these provide available forms of nutrients for plant assimilation (Krishna, 2002). Soil organic matter composed of mainly C and N is an important indicator for soil quality (Weil & Brady, 2002). Soils with an adequate amount of soil organic matter are fertile and productive, allowing for crop growth and development (Brevik, 2009). Soil C provides energy for microbial activities which generate organic and inorganic nutrients for plant acquisition,

especially N, an essential and limiting element for plant growth (Hopkins & Dungait, 2010). Therefore, managing soil fertility is crucial to sustain the productivity of cropping systems.

1.2 Crop productivity and soil nutrients

Crop productivity is largely dependent on soil nutrients in which N, phosphorus (P) and potassium (K) are three major elements necessary for plant growth and development. In addition to NPK, crops also require other important nutrients such as magnesium, sulfur and calcium and small quantities of trace elements including iron, zinc, boron, manganese, molybdenum (Bierman & Rosen, 2005). In modern farming systems, the availability of NPK to meet crop demands in soils is often improved by adding synthetic fertilizers (Cordell *et al.*, 2009, FAO, 2015).

Out of all the mineral nutrients, N is the most growth-limiting plant nutrient. N in the NO_3^- form is soluble and mobile in soils, thereby easily running-off from soils into surface and groundwater (Smil, 1999, Galloway *et al.*, 2003). Large amounts of N loss through leaching may lead to a substantial reduction in crop productivity (Syväsalo *et al.*, 2006). In addition, nutrients contained within plants are typically lost after plant harvest. Crop harvesting often results in a need to replenish soil nutrients to prevent soil deterioration, which can negatively influence crop yields (Hera, 1996). Thus, a large amount of synthetic N fertilizers (up to 112.9 million tonnes in 2015) has been applied to agro-ecosystems worldwide to maintain high crop productivity (FAO, 2015). The complex microbial communities within soils will drive the fertilizer-derived N transformations to provide plant-available NO_3^-, however this form of N can be easily lost from the soil (Bender *et al.*, 2015). Such N loss will be exacerbated if disturbances to the soils such as heavy rainfall occur, possibly resulting in negative effects on crop productivity (Sun *et al.*, 2008, Liang *et al.*, 2011). Therefore, the effective management of soil nutrients, particularly N, is key to maintaining productive agriculture (Hera, 1996, Goulding *et al.*, 2008).

1.2.1 Soil nitrogen cycling in agro-ecosystems

In an agroecosystem, soil N cycling plays an important role in providing inorganic N for crop uptake and contributing to N loss via gas emission and leaching. Five microbe-driven processes are crucial for soil N cycling, including N fixation, mineralization, immobilization, nitrification, and denitrification (Schepers & Raun, 2008). In addition, N leaching is also of particular interest due to its involvement in N loss from the plant-soil system (**Figure 1.1**).

Crops can assimilate NH_4^+ and NO_3^- produced from N fixation, mineralization and nitrification, whereas immobilization and denitrification perform opposite functions that convert inorganic N to organic forms and contribute to N loss through releasing N gases into the atmosphere (Winsor, 1958). Differing from natural ecosystems, human-managed cropping systems have relied heavily on the application of synthetic N fertilizers (Singh & Ryan, 2015).

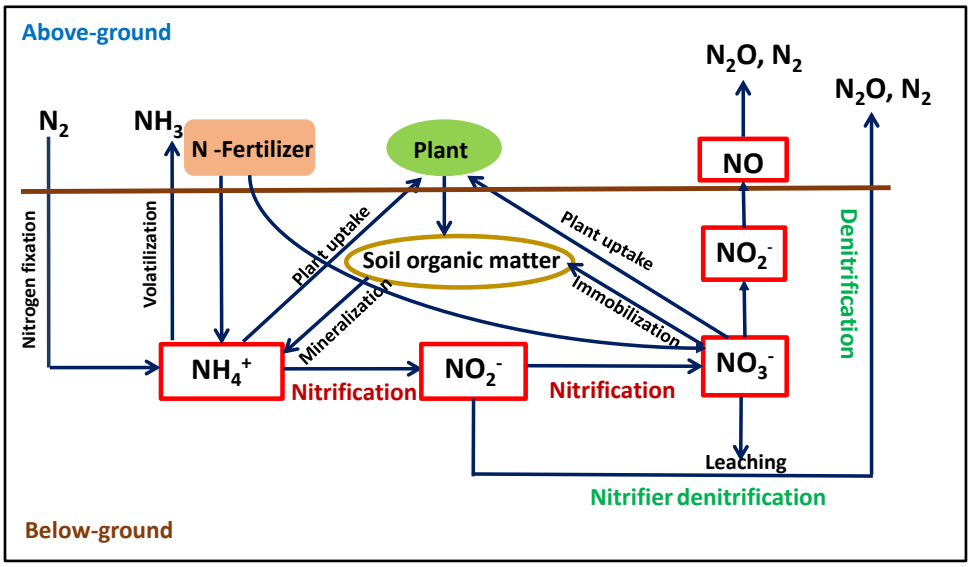

Figure 1.1 Soil N cycling in an agro-ecosystem (Adapted from the International Fertilizer Industry Association). NH_3 = Ammonia, NH_4^+ = Ammonium, NO_3^- = Nitrate, NO_2^- = Nitrite, NO = Nitric oxide, N_2O = Nitrous oxide, N_2 = Di-nitrogen, N = Nitrogen.

Addition of N fertilizers helps to fulfill the varying N requirements of crops at each developmental stage, although N made available by microbial activities including N-fixation and mineralization remains a key source of N for croplands. For example, cotton and cereal crops require the highest amount of N during the reproductive growth stage and rapid expansion phase to develop the crop canopy, which are critical stages of crop development (Mullins & Burmester, 2010, GRDC, 2005).

The application of N-fertilizers and the soil mineralization (ammonification) process both provide NH_4^+, the substrate for nitrification. Nitrification is a microbial process which generates NO_3^- necessary for plant uptake/assimilation to support plant growth and development. Nitrification produces the mobile compounds of nitrite (NO_2^-) and NO_3^-, which have the potential to be lost from soils due to their mobile nature through leaching (Conklin & Alfred, 2004). These compounds are also substrates of biological denitrification which releases N gases into the atmosphere. Such N loss through leaching and gas emission results in a reduction of plant available N in soils, therefore potentially negatively affecting crop productivity (Carlson, 2013).

In my PhD thesis, nitrification and denitrification are the primary focus since they have important roles in contributing to plant available N in soils and can be a cause of N loss through leaching into surface and ground water and gas emission into the atmosphere. Changes in these two processes may influence soil N availability which is directly related to crop productivity.

1.2.2 Soil nitrification and nitrifying communities

1.2.2.1 Nitrification process

In agricultural ecosystems, N-fertilizers have been commonly used in the ammonium form owing to its positive charges that can easily bind to soil particles, thereby to some extent avoiding N loss in run off (Ward *et al.*, 2011). Subsequently, ammonium fertilizers undergo the nitrifying process in soils to produce NO_3^- which is favorable for crop uptake, but more easily lost in wet soils. Soil nitrification consists of two types of processes

including autotrophic and heterotrophic nitrification. Autotrophic nitrification is considered a rate-limiting process requiring aerobic conditions and consists of two sequential steps: ammonia oxidation and nitrite oxidation (Bernhard, 2012). Ammonia oxidation is the process of converting NH_4^+ to NO_2^- via hydroxylamine by ammonia-oxidizing bacteria and archaea, subsequently NO_2^- will be converted to NO_3^- by nitrite-oxidizing bacteria (Bernhard, 2012) (**Figure 1.2**). In terms of heterotrophic nitrification, both bacteria and fungi have been found to possess genes encoding enzymatic pathways for oxidizing NH_4^+ or organic N to produce NO_3^- (**Figure 1.2**). The rate of heterotrophic nitrification, however, is 100 to 1000-fold lower than that of autotrophic nitrification (Lengeler *et al.*, 1999) and its quantitative importance in both aquatic and terrestrial ecosystems remains unresolved (Ward *et al.*, 2011).

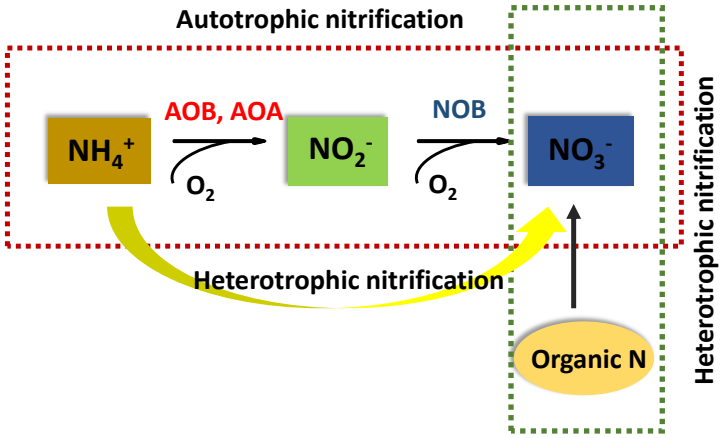

Figure 1.2 Autotrophic and Heterotrophic nitrification. Heterotrophic nitrification is conversion of either ammonia or organic N to nitrate. NH_4^+ = Ammonium, NO_3^- = Nitrate, NO_2^- = Nitrite, O_2 = Oxygen, N = Nitrogen, AOB = Ammonia-oxidizing bacteria, AOA = Ammonia-oxidizing archaea, NOB = Nitrite-oxidizing bacteria.

Since nitrification is a prerequisite for inorganic N loss from the plant-soil system, much attention has been given to controlling and managing the rate of nitrification. Similarly, efforts were made to establish a close relationship between the rate of nitrification and the rate of crop uptake in order to avoid N loss (Killham, 1994).

1.2.2.2 Nitrifying microorganisms

Soil nitrification is the microbe-driven process in which the first step, NH_3 oxidation, was thought to be carried out only by bacteria (Bernhard, 2012). But, a soil metagenomics study comprised of the analysis of chromosomal genes encoding metabolic pathways discovered archaea are also capable of oxidizing NH_3. The first archaeal isolate from a marine aquarium was reported in 2005 (Treusch *et al.*, 2005, Könneke *et al.*, 2005). Ammonia-oxidizing bacteria (AOB) are obligate autotrophs which generate energy for their growth using NH_3 to fix CO_2 through the Calvin Cycle (Ward *et al.*, 2011). AOB possess the *amoA* gene encoding ammonia monooxygenase (AMO) which catalyzes the NH_3 oxidation step (Purkhold *et al.*, 2000). AOB are Gram-negative and grow very slowly under laboratory conditions, resulting in a major limitation for obtaining pure cultures (Prosser, 2006). These chemolithotrophic AOB commonly belong to two major classes including the Beta- and Gamma-proteobacteria, in which representative strains cultivated so far are *Nitrosomonas* (Beta), *Nitrosospira* (Beta), and *Nitrosococcus* (Gamma) (Madigan *et al.*, 1997).

The first ammonia-oxidizing archaea (AOA) isolate from a marine aquarium, *Nitrosopumilus maritimus*, was initially assigned to the *Crenarchaeota* phylum (Stahl & de la Torre, 2012). AOA were then placed within the *Thaumarchaeota*, a new phylum suggested based on phylogenetic analyses (Stahl & de la Torre, 2012). AOA contain homologues of the AOB *amoA* gene encoding AMO for NH_3 oxidation catalysis. AOA have been demonstrated to be dominant in various ecosystems such as soils, oceans, and geothermal environments, suggesting a much wider range of habitats than their counterparts AOB (Karner *et al.*, 2001, Church *et al.*, 2003, He *et al.*, 2007, Zhang *et al.*, 2008, Reigstad *et al.*, 2008, Stahl & de la Torre, 2012). AOA representatives isolated so far from oceans, hot spring and soils including *Nitrosopumilus maritimus, Nitrosocaldus yellowstonii*, *Nitrososphaera gargensis*, *Nitrososphaera viennensis*, *Nitrosotalea devanaterra*, have provided evidence for their adaptation to harsh environments such as those containing extremely low NH_3 concentrations, acidic soils and high

temperatures (Martens-Habbena *et al.*, 2009, De la Torre *et al.*, 2008, Hatzenpichler *et al.*, 2008, Tourna *et al.*, 2011, Lehtovirta-Morley *et al.*, 2011).

AOB and AOA co-exist in agricultural soils and drive the first and rate-limiting step of nitrification, thereby affecting N fertilizer-use efficiency (Stark *et al.*, 2007). Changes in AOB and AOA activities and communities due to environmental impacts are expected to influence the nitrification rate, resulting in alterations to plant-available N levels (Rakshit *et al.*, 2012, Kelly *et al.*, 2011). Previous studies indicated potential niche differentiation between AOA and AOB that is shaped by differences in their NH_3 oxidation pathways and responses to environmental factors (Walker *et al.*, 2010, Erguder *et al.*, 2009, Yao *et al.*, 2013). These factors include substrate availability, soil pH, water content, temperature, precipitation, crop types and management practices (Fierer *et al.*, 2009, Erguder *et al.*, 2009). AOA often favors harsh environments such as low pH and substrate availability (Yao *et al.*, 2011, Hu *et al.*, 2016) whereas AOB becomes dominant in alkaline conditions and/or high NH_3 concentration (Verhamme *et al.*, 2011, Shen *et al.*, 2008).

Although AOA have been frequently reported to outnumber AOB in soils (Leininger *et al.*, 2006, Yao *et al.*, 2013, Hu *et al.*, 2015), uncertainties regarding their relative contribution to nitrification remain (Prosser & Nicol, 2012, Banning *et al.*, 2015). The numerical dominance of AOA was not always equivalent to the expression of their functions. For example, AOB was found to be less abundant than their counterparts AOA in some agricultural soils, but they were functionally dominant in the process of nitrification, with >76.7% contribution to nitrification activity (Jia & Conrad, 2009, Xia *et al.*, 2011). In addition, changes in the rate of nitrification have been reported in the relationship with microbial population kinetics in a number of studies (Horz *et al.*, 2004, Hu *et al.*, 2016, Hu *et al.*, 2015, Liu *et al.*, 2015). Thus, impacts of different environmental factors on nitrification rates are often hard to discern, leading to difficulties in N source management for crop growth and development. More studies outlining nitrifying communities and their interactions with environmental factors are needed to provide a comprehensive understanding of the responses of microbial nitrification to

environmental changes in agro-ecosystems, to support sustainable agricultural productivity.

In addition to AOB and AOA, characterization of nitrite-oxidizing bacteria (NOB), which drive the second step of nitrification (**Figure 1.2**), is also necessary. However, the lack of a technical NOB universal 16S rRNA gene probe has limited the understanding of the ecological importance of NOB in soil environments (Freitag *et al.*, 2005). NOB possesses nitrite oxidoreductase (Nxr) to catalyze the conversion of nitrite to nitrate, which can be utilized by crops (Bernhard, 2012). This process contributes to energy production for NOB; however, the amount of energy generated is small, resulting in very slow growth of NOB (Bernhard, 2012). As a result, it is difficult to obtain pure cultures of NOB for kinetic studies. Based mainly on cell morphology and membrane ultrastructure, NOB is divided into four genera: *Nitrobacter*, *Nitrococcus*, *Nitrospina*, and *Nitrospira*, in which only *Nitrobacter* have been studied in terms of biochemistry and physiology (Prosser, 2006). It was thought that *Nitrobacter* functionally dominates the nitrification process; however, increasing evidence suggests the important role of *Nitrospira* in nitrification (Xia *et al.*, 2011). The molecular mechanism of bacterial nitrite conversion is still not completely understood (Cabello *et al.*, 2009).

In addition to autotrophic nitrifiers, heterotrophic nitrifiers with a wide phylogenetic range, have been reported to be capable of nitrifying by oxidation of either organic or inorganic N compounds (De Boer & Kowalchuk, 2001, Nishio *et al.*, 1998, Daum *et al.*, 1998, Hora & Iyengar, 1960). These heterotrophic nitrifiers include bacteria and fungi with some representatives including *Pseudomonas putida*, *Alcaligenes faecalis, Thiosphaera pantotropha, Aspergillus* and *Penicillium*. Heterotrophic nitrifiers are known to be important under conditions that are unfavorable for autotrophic nitrifiers (Brierley & Wood, 2001). Previous studies have often reported the functional role of heterotrophic nitrifiers in grassland and forest soils (Well *et al.*, 2008, Xu & Inubushi, 2005, Du *et al.*, 2006).

1.2.3 Soil denitrification and denitrifying communities

1.2.3.1 Denitrification process

Denitrification is a microbial respiratory process by which NO_3^- or NO_2^- is used as an alternative electron acceptor when oxygen (O_2) is limiting and reduced into gaseous N either as molecular N_2 or as an oxide of N (Knowles, 1982). This process is sequentially catalyzed by different enzymes including nitrate reductase (*Nar* and *Nap*), nitrite reductase (*Nir*), nitric oxide reductase (*Nor*), and nitrous oxide reductase (*Nos*) (Cabello *et al.*, 2009) (**Figure 1.3**).

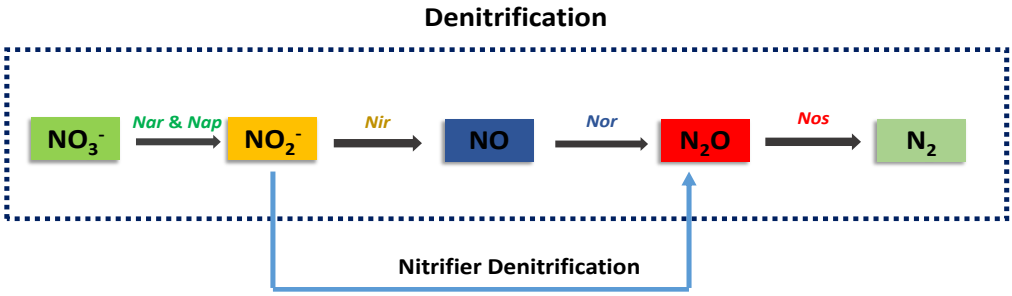

Figure 1.3 Pathway and enzymes involved in denitrification. NO_3^- = Nitrate, NO_2^- = Nitrite, NO = Nitric oxide, N_2O = Nitrous oxide; N_2 = Di-nitrogen, *Nar* = Nitrate reductase, *Nap* = Nitrate reductase, *Nir* = Nitrite reductase, *Nor* = Nitric oxide reductase, *Nos* = Nitrous oxide reductase.

1.2.3.2 Denitrifying microorganisms

A large number of soil prokaryotes are involved in denitrification, which is reported to account for 5% of the total soil microbial community (Tiedje, 1988). Denitrifiers are facultative anaerobic organisms that can use N oxides as electron acceptors when O_2 is limited. Approximately 60 genera of denitrifying microorganisms have been reported to belong to proteobacteria, but additional denitrifiers include halophilic and hyperthermophilic archaea, and fungi (Philippot *et al.*, 2007). It is interesting that several

isolated strains participating in nitrification or N fixation can also express denitrifying activity; e.g., *Nitrosospira* and *Nitrosomonas* are capable of denitrification (Shaw *et al.*, 2006).

Denitrification is of particular interest to farmers since N loss from cropping systems can occur via this process, by which plant-available N in soils is returned to the non-available atmospheric N pool (Freney & Simpson, 2013). Denitrification is highly variable in time and space in cropping soils due to factors controlling the rate of the process (Philippot *et al.*, 2007). These factors mainly include soil properties, climate and management practices including crop species selection, fertilizer type and N-fertilizer rate (Hofstra & Bouwman, 2005). Since denitrification is a microbially-mediated process, changes in denitrification rates are assumed to be linked with altered physiology or population dynamics of denitrifiers (Schimel & Gulledge, 1998). Despite an increasing number of studies on the relationship of "ecosystem functioning-microbial communities", the underlying correlation between denitrification rate responses to environmental impacts and denitrifying communities remains debatable (Braker *et al.*, 2010). For example, changes in denitrification rates in response to fertilizer addition and reduced N deposition in an agricultural field trial and a spruce forest soil, respectively resulted from shifts in denitrifying bacterial communities (Hallin *et al.*, 2009, Kandeler *et al.*, 2009). No correlation between denitrification rates and denitrifying communities was observed in several studies on agricultural soils (Miller *et al.*, 2008, Wertz *et al.*, 2009). Thus, a complete understanding of whether and how denitrifying community structure is affected by changes in controlling factors may help to develop strategies to minimize N loss from the plant-soil systems, supporting sustainable agricultural productivity.

1.3 Crop productivity and soil processes under global change

Climate model projections have indicated a doubling in atmospheric CO_2 concentration and a 1.1 to 6.4^{o}C increase in global average temperatures during the 21st century (IPCC, 2013). Additionally, extreme weather events including heat waves, prolonged-drought and flooding have been predicted to increase in frequency and intensity (Pachauri &

Reisinger, 2007). The agricultural sector is expected to be highly sensitive and substantially affected by such global change (Howden *et al.*, 2007, Lesk *et al.*, 2016). Warming and elevated CO_2 can markedly influence crop growth and productivity through alterations in crop physiology and soil processes (Reddy & Hodges, 2000). Previous studies have predominantly reported increases in crop yields under increases in atmospheric CO_2 levels (Taub *et al.*, 2008, De Souza *et al.*, 2008) while elevated temperatures could either reduce or enhance crop productivity, depending on the elevated temperature in relation to the plant optimum growth temperature (Rustad *et al.*, 2001, Lobell & Field, 2007, Asseng *et al.*, 2015).

Potential changes in soil processes in response to increasing CO_2 and temperature can result in alterations in soil nutrient availability, particularly N which is directly related to crop productivity (Brevik, 2012, Kang *et al.*, 2009). For example, soil N mineralization often responds positively to warming (Bai *et al.*, 2013), thereby possibly enhancing plant-available N. A study in wheat farms by Rakshit *et al.* (2012) reported an increase in N mineralization under CO_2 fertilization whereas no effect of elevated CO_2 on this process was observed in a study of soybean and sorghum (Henning *et al.*, 1996). Management of soil N availability in agro-ecosystems under climate change may be hard in part due to large amounts of N-fertilizer supply and variations in N requirements of crops and different developmental stages. In addition, simultaneously increasing both CO_2 and temperature may not have an additive effect on crops or soil nutrient levels (Sun *et al.*, 2012, Liu *et al.*, 2015). Thus, a complete understanding of how soil N processes respond to climate change will help develop effective N management strategies which can support sustainable agricultural production.

Extreme weather events can severely affect agricultural productivity (Pachauri & Reisinger, 2007). Approximately 20-30 million hectares of agricultural land worldwide have suffered waterlogging due to intensified rainfall (Setter & Waters, 2003, FAO, 2007). Drought due to inadequate and poorly distributed rainfall has also caused a reduction in crop production (Toulmin, 1987, Gopinath & Bhat, 2012). For example,

Australian agriculture suffered a loss of about $6 billion in grain profitability due to a severe flooding event in 2010-2011 (Boote *et al.*, 2011). In addition, it is estimated that over 50% of croplands worldwide may suffer drought stress in the next 50 years (Sinclair, 2011). Flooding and drought can have negative impacts on crop physiology and soil processes regulating nutrient cycling, leading to reduction in crop productivity (Li *et al.*, 2016, Jaleel *et al.*, 2009, Al-Kaisi *et al.*, 2013). For example, soil N availability may be significantly depleted due to stimulated leaching and denitrification upon flooding events (Irmak & Rathje, 2008). Such impacts on soil N processes may establish a legacy of extreme weather events which can influence crop productivity in subsequent years (Cavagnaro, 2016). Hence, it is imperative to elucidate whether there is establishment of the legacy of extreme weather events. This will help to develop N management strategies to maintain crop productivity in the context of higher frequency and magnitude of extreme weather events.

1.3.1 Response of crop productivity to elevated temperature and CO_2

Temperature can alter crop productivity through impacts on the length of crop duration, water availability and plant physiological processes such as photosynthesis and respiration (Lobell & Gourdji, 2012). Each plant species possesses their own range of the optimal temperatures for growth and development (Hatfield *et al.*, 2011). Hence, the response of crop productivity to increasing temperatures will be dependent on crop type and the magnitude of temperature change. Challinor *et al.* (2005) and Ferris *et al.* (1998) observed a substantial reduction in the yield of peanut and wheat crops after short-term exposure to temperatures higher than their relative optima at the flowering stage; due to increased pollen sterilization. In another scenario, higher temperatures could accelerate plant growth and development, resulting in a shorter growing season that may affect crop yields (Stone, 2000). The productivity of staple crops such as wheat has been reported to decrease when crop duration was shortened (Wheeler *et al.*, 2000, Hatfield *et al.*, 2011). Additionally, warming exponentially increases saturation vapor pressure of the air, thereby increasing the difference between saturated and actual air

vapor pressure, and consequently reducing relative humidity and increasing vapor pressure deficit (VPD). This could negatively affect plant water-use efficiency by increasing water loss per unit of carbon gain (Ray *et al.*, 2002). In terms of crop physiology, elevated temperature can have positive or negative impacts on physiological processes, particularly on photosynthesis. The rate of photosynthesis will increase if the new temperature is closer to the plant optimum temperature value, and decrease if the new temperature is above the optimum. For example, the study by Bibi *et al.* (2008) found a significant decline in the photosynthetic rate of cotton plants when the temperature exceeded 35^{o}C. Elevated temperatures may negatively alter boll development, reducing cotton lint yields if they are much higher than the optimum range, depending on the limitation of other factors (Reddy *et al.*, 1999).

Crops generally respond positively to elevated CO_2 through increased biomass production, photosynthesis and water use efficiency (Kimball *et al.*, 2002). Crops can get benefits when exposed to CO_2 fertilization since C fixation by ribulose bisphosphate carboxylase oxygenase (or Rubisco) is favored due to the increasing CO_2:O_2 ratio (Ziska & Bunce, 2006). Previous studies have reported an increase in crop biomass and productivity due to stimulated photosynthetic rate. For example, a 40% increase in the dry vegetative mass of cotton was observed when the CO_2 level was doubled to 720 ppm, which was likely due to increased photosynthetic rates (Reddy *et al.*, 1997). The biomass and yield of cotton grown in free-air CO_2 enrichment (FACE) system with 550 ppm of CO_2 level increased 37% and 43%, respectively (Mauney *et al.*, 1994). De Souza *et al.* (2008) also found 30% and 40% increases in biomass and photosynthetic rate of sugarcane in response to an elevation of CO_2 from 370 ppm to 720 ppm, respectively, thereby increasing crop productivity approximately 29%. Elevated CO_2 may reduce plant transpiration due to decreasing stomatal conductance, consequently leading to increased water use efficiency (Varga *et al.*, 2015). O'Leary *et al.* (2015) observed that water use efficiency for wheat biomass and productivity increased 36% and 31%, respectively at a CO_2 concentration of 550 ppm. Cotton water use efficiency was also observed to increase due to the increased above-ground biomass (Mauney *et al.*, 1994).

Additionally, CO_2 fertilization has been indicated to increase the productivity of some crops such as rice and tomato through other mechanisms including enhanced hormone concentrations and ethylene production which affect plant growth and development (Smith & John, 1993, Sisler & Wood, 1988, Wooddrow & Grondzinski, 1993, Seneweera *et al.*, 2003). For example, Seneweera *et al.* (2003) found that rice grown under CO_2 enrichment produced higher yields due to enhanced ethylene production which promoted tiller appearance and the release of auxiliary buds.

Effects of elevated temperature in combination with elevated CO_2 on crop productivity have been not fully understood (Sun *et al.*, 2012). Since many studies have focused on effects of single climate factor on crop yields (Wheeler *et al.*, 2000, Hatfield *et al.*, 2011, De Souza *et al.*, 2008, Seneweera *et al.*, 2003, Zhang *et al.*, 2013, Burkey *et al.*, 2007, Yoshida *et al.*, 2011, Bunce, 2016). Despite the importance of the interaction between elevated CO_2 and temperature, fewer studies have investigated crop productivity response to combined elevated CO_2 and temperature. Elevated CO_2 has been shown to ameliorate the negative effects of elevated temperature on crop yields (Mohanty *et al.*, 2015, Figueiredo *et al.*, 2015). However, no beneficial effects of combined elevated CO_2 and temperature on the yield of several crops such as rice, soybean, dry bean, peanut, cowpea, wheat and cotton were also observed (Matsui *et al.*, 1997, Baker *et al.*, 1989, Prasad *et al.*, 2002, Prasad *et al.*, 2003, Ahmed *et al.*, 1993, Wheeler *et al.*, 1996, Reddy *et al.*, 2000). Thus, the combination of increased atmospheric CO_2 and temperature may not have additive effects on crop yields.

Overall, increasing temperatures can stimulate crop productivity mainly through higher photosynthetic rates and longer growing seasons; however, this will depend on the crop type and magnitude of temperature increase. Regarding CO_2 fertilization, crop productivity is often enhanced via the stimulation of photosynthesis, water use efficiency and plant biomass. As simultaneously increasing CO_2 and temperature may not additively affect crop productivity (Sun *et al.*, 2012, Cai *et al.*, 2016), more studies to investigate interactive impacts of future climate factors on crop yields are required.

The availability of limiting resources such as soil nutrients and water affects plant responses to elevated CO_2 and temperature (Rustad *et al.*, 2001, Reich *et al.*, 2006, Derner *et al.*, 2003, Reich *et al.*, 2014). For example, tundra plant growth significantly increased under climate warming in the first 3 years, and then did not change in the 4th year possibly due to soil nutrient depletion (Arft *et al.*, 1999). Adding nutrients to a forest soil made CO_2-induced carbon sequestration apparent in the study by Oren *et al.* (2001). In agro-ecosystems, the relationship between crop response to elevated CO_2 and soil nutrient status, particularly N, has also been reported in some studies (Li *et al.*, 2003, Franzaring *et al.*, 2011). These studies pointed to a reduction in the response of crop growth and yields to elevated CO_2 when soil N availability was low. Elevated temperature and CO_2 can directly and indirectly influence soil processes which mediate nutrient cycling, thereby potentially affecting soil nutrient availability (Hu *et al.*, 2016, Bardgett *et al.*, 2008). As a result, the magnitude and direction of crop productivity response to elevated CO_2 and temperature may be altered. Given N fertilizer supply and variation in N requirements between different crops and different developmental stages in agricultural systems, the effects of elevated CO_2 and temperature on crop productivity through their impacts on soil nutrient availability remains largely unknown. Thus, we need a complete understanding of how future climatic conditions affect soil nutrients and consequences for crop productivity.

1.3.2 Response of soil nitrification and denitrification to elevated temperature and CO_2

1.3.2.1 Response of soil nitrification

Elevated temperatures are expected to accelerate the rates of many biological processes in soils, particularly nitrification (Melillo *et al.*, 2002, Yun *et al.*, 2011). Elevated temperatures can directly affect nitrification processes through stimulating metabolic activities of nitrifiers whose enzyme-mediating reactions are sensitive to temperature (Hu *et al.*, 2016, Karhu *et al.*, 2014). Additionally, soil and plant properties may respond to elevated temperature, resulting in alterations in soil substrate availability which

controls the ammonia-oxidation step (Hu *et al.*, 2016). In particular, increasing temperatures often stimulate N mineralization, litter decomposition and soil respiration, thereby potentially changing soil NH_4^+ concentration and O_2 status, respectively (Bai *et al.*, 2013, Rustad *et al.*, 2001, Bradford *et al.*, 2008). Some studies also found significant changes in the amount of root exudation and plant biomass in response to climate warming, possibly leading to increased availability of C and N sources in soils (Uselman *et al.*, 2000, Yin *et al.*, 2013). Alternatively, elevated temperature may alter nitrification via re-structuring ammonia-oxidizing communities, resulting in modifications of physiological properties which drive the process rate (Schimel & Gulledge, 1998). However, we still do not know whether and how nitrifying communities are affected by increased temperatures.

The response of nitrification to elevated temperatures has been suggested to be ecosystem-specific (Bai *et al.*, 2013). For example, nitrifying pure cultures are believed to perform their optimal growth in the temperature range of 25-30^{o}C; however, increased temperatures close to this range did not change nitrifying enzyme activities and net nitrification of tropical soils in a meta-analysis study by Barnard *et al.*, (2005) . In contrast, the study by Hu *et al.* (2016) found significant increases in the nitrification rate under climate warming for dryland soils. Such discrepancies may be attributed to differences in soil abiotic properties, microbial communities, and climatic conditions of sites. In high yielding agricultural systems, nutrients and water supply are often optimized, suggesting a unique response of soil nitrification to climate warming. Very few studies on the nitrification response to climate warming in agricultural soils have been conducted so far. The limited number of studies has reported inconsistent data. For example, elevated temperatures were shown to decrease the nitrification rate and did not affect ammonia-oxidizers in a study investigating wheat (Rakshit *et al.*, 2012), while research by Liu *et al.* (2015) showed no effects on both nitrification rates and ammonia-oxidizers of a field study using rice. The differences in results could be attributed to the different crop species investigated, differences in soil properties,

sampling date and/or climatic conditions of each site. Hence, the nitrification response to climate warming in agricultural soils remains largely unknown.

Elevated CO_2 may only have indirect impacts on nitrification through its effect on plants, since it minimally affects the CO_2 concentration in soils (Singh *et al.*, 2010, Kelly *et al.*, 2013, Johnson *et al.*, 2001). Thus, CO_2 enrichment is expected to alter nitrification rates via plant-mediated changes in soil properties and nitrifiers. CO_2 fertilization has been indicated to increase labile C input to soils due to increased plant root biomass, root exudates and turnover of fine roots (Cardon *et al.*, 2001, Sadowsky & Schortemeyer, 1997, Zak *et al.*, 1993). Such increases may stimulate other biological processes such as N mineralization, resulting in alterations in soil NH_4^+ concentration (Hungate *et al.*, 1999). Elevated CO_2 also may accelerate plant N uptake, potentially leading to an increase in the competition between NH_4^+ consumers such as nitrifiers and plants (Hungate *et al.*, 1997). In addition, soil water content may increase as a result of CO_2-induced plant responses, thereby possibly changing soil O_2 concentrations and hence affecting nitrification efficiency (Barnard *et al.*, 2006). Alternatively, elevated CO_2 may re-structure ammonia-oxidizing communities, resulting in changes in physiologies which control the rate of nitrification. However, the underlying mechanism of the nitrifier response to CO_2 fertilization remains unclear.

In nutrient-poor ecosystems, soil nitrification has been reported to decrease in response to elevated CO_2 (Hungate *et al.*, 1997, Lagomarsino *et al.*, 2008, Barnard *et al.*, 2006). Explanations could be due to lower O_2 status caused by increased soil water content (Barnard *et al.,* 2005), or increased competition for NH_4^+ among nitrifiers, plants and heterotrophic microorganisms (Hungate *et al.*, 1997, Lagomarsino *et al.*, 2008). High yielding cropping systems are often well-watered and N-fertilized; and hence, soil nitrification may respond differently to elevated CO_2. There are a limited number of studies on soil nitrification response to elevated CO_2 in agricultural systems, and these studies have reported contradictory evidence (Pereira *et al.*, 2011, Pereira *et al.*, 2013, Liu *et al.*, 2015). For example, no changes were observed in the AOB and AOA

communities and nitrification rates under CO_2 fertilization in soybean fields whereas the opposite trend was observed in a rice field study (Pereira *et al.*, 2011, Pereira *et al.*, 2013, Liu *et al.*, 2015). Such discrepancies may be attributed to the interaction of elevated CO_2 and other factors such as crop species, soil types and climatic conditions when samples were taken.

Overall, a complete picture of the nitrification response to single factors such as elevated CO_2 and temperature and the combination of both factors in agricultural soils is required since interactive effects of elevated CO_2 and temperature on nitrification may not be additive (Hu *et al.*, 2016, Liu *et al.*, 2015). Consequently, the role of soils in mediating a crop productivity response to future climate conditions will be fully understood, thereby allowing the development of effective N management to maintain high crop yields.

1.3.2.2 Response of soil denitrification

Understanding denitrification responses to increasing temperature is of great significance to the management of plant-available N in agricultural soils. The denitrification process is favored in poorly aerated soil conditions with high labile C and NO_3^- availability, and neutral pH (Barnard *et al*., 2005). Elevation of temperature may directly or indirectly affect denitrification by influencing denitrifying communities and plant and soil properties (Braker *et al.*, 2010, Saad & Conrad, 1993, Bremer *et al.*, 2009). Plant root exudates have been shown to increase in some studies (Uselman *et al.*, 2000, Yin *et al.*, 2013), thereby increasing labile C inputs into soils. Such increases in soil carbon are favored by denitrifiers (Hofstra & Bouwman, 2005). Elevated temperature often increases soil respiration and N mineralization (Rustad *et al.*, 2001), possibly resulting in an increase in N substrate and a decrease in O_2 availability which are favorable conditions for denitrifiers. Increased oxygen consumption in the soils results from enhanced microbial respiration caused by elevated temperature, leading to a favorable environment for denitrifying bacteria, which potentially increases the rate of denitrification (Castaldi, 2000). Veraart *et al.* (2011) reported a doubling in the denitrification rate when the temperature was 3^{o}C higher than under ambient conditions

in both field and microcosm experiments due to decreased O_2 levels. On the other hand, elevated temperatures may affect multiple soil processes and increase plant biomass and N uptake, resulting in overall small effects or decreased NO_3^- sources for denitrifiers (Dijkstra *et al.*, 2012).

The denitrification rate has been often reported to be positively correlated with elevated temperatures (Gödde & Conrad, 1999, Maag & Vinther, 1996, Bailey, 1976, Braker *et al.*, 2010). Given it is a microbially-mediated process, elevated temperature can directly alter microbial physiologies, or indirectly re-structure denitrifying communities, leading to changes in overall denitrification kinetics (Schimel & Gulledge, 1998). However, the underlying mechanism of denitrifier response to elevated temperature remains largely debatable. The question here is whether changes in the denitrifying community are reflected in their functions. Previous studies found contrasting evidence of the "denitrifying community-functioning" relationship across various ecosystems (Hallin *et al.*, 2009, Kandeler *et al.*, 2009, Wertz *et al.*, 2009, Miller *et al.*, 2008, Dandie *et al.*, 2008). For example, Bremer *et al.* (2009) observed a significant correlation of the abundance and structure of denitrifying communities and their function in a grassland soil, whereas Boyle *et al.* (2006) and Wertz *et al.* (2009) could not find such correlations in forest and agricultural soils, respectively. It has been suggested that the combination of denitrifying communities and their expressed functions depends on ecosystem and not yet understood environmental factors (Rich & Myrold, 2004, Wallenstein *et al.*, 2006a).

It has been shown that elevated CO_2 has only a modest effect on soil CO_2 concentration (Johnson *et al.*, 2001). As a result, elevated atmospheric CO_2 is expected to alter soil denitrification via CO_2-induced plant responses (Kelly *et al.*, 2013). Denitrification may be favored under elevated CO_2 concentrations due to an increase in soil labile carbon sources resulting from increased root exudations, root biomass and turnover of fine roots (Cardon *et al.*, 2001, Sadowsky & Schortemeyer, 1997, Zak *et al.*, 1993, Rogers *et al.*, 1998). Additionally, soil O_2 concentration may decrease due to the stimulation of soil respiration and plant water use efficiency under elevated CO_2 (Zak *et al.*, 2000, Bunce,

2004). Such declines in soil O_2 levels are also favorable for denitrification (Barnard *et al*., 2005). Moreover, soil NO_3^- availability for denitrifers may be depleted when plants are exposed to elevated CO_2 since plant N uptake can be accelerated, thereby negatively influencing denitrification rates (Hungate *et al.*, 1997, Kelly *et al.*, 2013, Hu *et al.*, 2001).

In non-cropping systems without an external N supply, denitrification has often been reported to be unresponsive or decreased under CO_2 elevation (Matamala & Drake, 1999, Barnard *et al.*, 2004, Barnard *et al.*, 2005). This could be explained by the limitation of N availability in the soils due to an increase in plant N demands under CO_2 enrichment (Hungate *et al.*, 1997, Mosier *et al.*, 2002). It has been suggested that denitrification will increase in response to elevated CO_2 only when N-fertilizer is applied in excess of plant N demand (Dijkstra *et al.*, 2012). Thus, soil denitrification may positively respond to elevated CO_2 in high yielding cropping systems where water and N supply are often optimized.

Given that denitrification is a microbially-mediated process, the underlying mechanism of changes in drivers in response to elevated CO_2 remains largely unknown. The question here is whether and how the denitrifying community structure is affected by CO_2-induced plant responses and the consequences for rates of denitrification. Evidence provided from previous studies are highly variable across different ecosystems. For instance, Deiglmayr *et al.* (2004) did not find any correlation between denitrification rate and denitrifying community in a grassland ecosystem, whereas denitrifying activity and community structure were significantly correlated in a forest study by Rich *et al.* (2003). Such discrepancies have been suggested to be due to ecosystem-specific factors and other not yet understood environmental factors (Rich & Myrold, 2004, Wallenstein *et al.*, 2006a).

Despite an increasing number of studies on the response of soil denitrification to climate change, little is known about the combined impacts of elevated CO_2 and temperature on this process, particularly whether they are additive, synergistic, or antagonistic than the sum of each single effect alone. Thus, a complete understanding of the soil denitrification

response to elevated CO_2 and temperature in agroecosystems is required and will help to minimize N loss from the plant-soil systems, thereby sustaining high crop yields under future climatic conditions.

1.3.3 Response of crop productivity to extreme weather events

1.3.3.1 Response of crop productivity to flooding

Flooding, resulting in soil waterlogging and even complete submergence of plants, is a major abiotic stress which severely affects crop growth and development, leading to substantial reductions in crop productivity worldwide (Setter & Waters, 2003, FAO, 2007). Flooding quickly results in the depletion of O_2 concentration from soils, particularly at a few millimeters of depth, due to the lower diffusion rate of O_2 in water than in air and the O_2 consumption of soil microbes and plant roots (Visser *et al.*, 2003, Armstrong & Drew, 2002). The O_2 deficiency can significantly reduce crop yields through limiting plant photosynthesis, respiration, nutrient uptake, and even causes plant death if the essentially aerobic respiration of roots completely ceases (Huang *et al.*, 2003, Bailey-Serres & Colmer, 2014, Guang *et al.*, 2012, Milroy *et al.*, 2009). However, the magnitude of crop productivity responses to flooding depends on crop growth stage, the depth of water levels and the duration of flooding events (Watson *et al.*, 1976, Malik *et al.*, 2001, Jackson, 1979). For example, Bange *et al.* (2004) reported that waterlogging events at the early reproductive stage caused larger losses in cotton yield than at later growth stages.

As soil environments can be hypoxic or even anoxic upon flooding, the duration of plants capable of resisting such unfavorable conditions depends on individual species (Visser *et al.*, 2003). Flooding mainly limits the supply of O_2 for plants, however, some plants can modify morphological and biochemical characteristics to improve their access to O_2 resources, including the development of aerenchyma, increases in the shoot elongation rate, the synthesis of anaerobic proteins, and the prevention of potential phytotoxin productions (Colmer, 2002, Vrienzen *et al.*, 2003, Subbaiah & Sachs, 2003, Dordas *et al.*,

2003). For example, rice crops have been known to be highly tolerant to flooding since they can develop lysigenous aerenchyma and a barrier to radial O_2 loss (Nishiuchi *et al.*, 2012). Flooding generally results in higher rice yields when compared to saturated or aerobic conditions (Satyanarayana & Ghildyal, 1970). In contrast, some crops such as cotton and wheat are poorly adapted to waterlogging and submergence (Najeeb *et al.*, 2015, Malik *et al.*, 2001). These crops suffer a series of deleterious symptoms caused by flooding stress such as reduced photosynthesis and root metabolism, resulting in a substantial reduction in crop productivity (Bange *et al.*, 2004, Shao *et al.*, 2013). For example, wheat grain yields reduced by 40% and 53% due to intermittent and continuous waterlogging events for 42 days, respectively (Watson *et al.*, 1976). Similarly, a 27-30% reduction of cotton yield was observed for plants subjected to waterlogging for 4-9 days during the early reproductive stage (Wu *et al.*, 2012).

Crop productivity responses to flooding depend on soil nutrient availability, especially N (Huang *et al.*, 1994, Harris *et al.*, 2016). Soil N availability is expected to decrease in response to water stress due to the possible enhancement of NO_3^- leaching and denitrification in soils (Unger *et al.*, 2009), thereby possibly exacerbating the crop response to flooding. Given the N-fertilizer supply in agricultural systems and the varied N uptake of different developmental stages in crops, the extent to which soil mediates the crop productivity response to flooding remains relatively unknown.

1.3.3.2 Response of crop productivity to drought

Drought events are the main abiotic stress severely constraining crop growth and development, resulting in negative effects on crop productivity (Lambers *et al.*, 2008). Drought rapidly limits soil water availability and increases the atmospheric evaporation demand (Jaleel *et al.*, 2009). Crop productivity responds to drought conditions mainly through morphological and genetic modifications, and alterations in biochemical-physiological processes and nutrient uptake (Xu *et al.*, 2010). Faced with water deficit stress, crop plants are likely to endure a series of adverse events including a decrease in growth rates (Nonami, 1998, Manickavelu *et al.*, 2006) leaf water potential, relative

water content and transpiration rates (Nerd & Nobel, 1991, Siddique *et al.*, 2000), root acquisition of nutrients and their transport to shoots (McWilliams, 2003, Farooq *et al.*, 2009), photosynthesis (Bota *et al.*, 2004, Wahid & Rasul, 2005), root respiration (Liu & Li, 2005), and the stimulation of reactive oxygen species formation (Blokhina *et al.*, 2003, Reddy *et al.*, 2004) and even fatal consequences. For example, Escalona *et al.* (2000) observed a 59% decrease in the yield of field-grown grapes exposed to severe prolonged-drought periods due to the dramatic decline in photosynthetic rates. Similarly, cotton lint yield reduced by 31-35% and 57-60% due to a decline in boll number and size when soil water levels were 60% and 45% of field capacity, respectively, compared to 75% of field capacity (Wang *et al.*, 2016).

Despite potential drought impacts on crops ranging from moderate to extremely severe, crop plants may display unique adaptation strategies to alleviate these effects (Xu *et al.*, 2010). These adaptation strategies include (i) escape from drought by shortening the life cycle or growing season before the onset of severe stress, (ii) drought avoidance to minimize water loss from plants and enhance plant water uptake through dense leaves and increased root to shoot ratio, (iii) drought tolerance through physiological and molecular mechanisms including water conservation of cells and tissues by osmotic adjustment and changes in cell wall elasticity, altered metabolic pathways by such as enhancement of the activity of antioxidant compounds, and genetic mutation and modification (Farooq *et al.*, 2009, Xu *et al.*, 2010). For example, some crops such as upland rice, tea, onion, legume and cotton can elongate their root systems to allow them to access deep water in the soils under water deficit stress (Subbarao *et al.*, 1995, Kavar *et al.*, 2008, Turner *et al.*, 2001, Iijima *et al.*, 2007), thereby potentially diminishing the negative impacts of drought periods on productivity.

The magnitude of crop productivity responses to drought depends on the duration and severity of drying-rewetting cycles and crop phenology (Farooq *et al.*, 2009). Most crops are strongly vulnerable to drought stress during reproductive growth (Lafitte *et al.*, 2007, Martínez *et al.*, 2007, Samarah *et al.*, 2006, Kamara *et al.*, 2003). For example, Pettigrew

(2004) observed a significant lint yield loss in cotton exposed to a higher intensity of drought stress during reproductive growth. Similarly, severe water deficit stress during reproductive growth caused up to a 94% reduction of rice yield whereas only 60% was observed at the grain filling stage (Lafitte *et al.*, 2007, Basnayake *et al.*, 2006).

1.3.4 Response of soil nitrification and denitrification to extreme weather events

1.3.4.1 Response of soil nitrification and denitrification to flooding

The depletion of soil O_2 is unfavorable for nitrifiers, and hence the rate of nitrification is expected to decrease in response to flooding conditions (Reddy & Patrick, 1975). Previous studies have provided contradictory evidence of the nitrification response to flooding (Vlek & Craswell, 1981, Wild & Cameron, 1981, Engelaar *et al.*, 1995, Zia *et al.*, 2001). For example, the rate of nitrification significantly decreased when soils were subjected to waterlogging due in part to the elimination of air by water (Zia *et al.*, 2001). In contrast, Engelaar *et al.* (1995) observed no changes in nitrification rates of waterlogged soils planted with *Rumex palustris*. Such maintenance of nitrification rates was due to the radial O_2 loss from aerenchymatous roots to the rhizosphere (Engelaar *et al.*, 1995, Laan *et al.*, 1989).

Under waterlogging conditions, nitrification efficiency is driven by the competition between plants and microorganism for NH_4^+ (Engelaar *et al.*, 2000, Zia *et al.*, 2001). Thus, the nitrification rate response to flooding will be negative if O_2 shortage occurs or plants and/or other microbes are superior competitors for NH_4^+. Given that nitrification is a microbe-driven process, little is known about whether and how the nitrifying community is involved in the nitrification response to flooding. It has been suggested that shifts in microbial community abundance and structure may lead to changes in ecosystem functions (Schimel & Gulledge, 1998). Changes in soil substrate availability and other soil properties under waterlogging conditions may lead to differential responses of AOB and AOA. However, little is known about the role of the nitrifying community in mediating the process response to flooding events, particularly in agricultural soils.

Following O_2 depletion resulting from flooding events, denitrifers (facultative anaerobic microorganisms utilizing NO_3^- as the alternative terminal electron acceptor) are stimulated (Megonigal *et al.*, 2005). As a result, the rate of denitrification often increases upon flooding (Burford & Bremner, 1975, Bronson & Fillery, 1998, Engelaar *et al.*, 2000, Hamonts *et al.*, 2013). Denitrification efficiency is mainly affected by soil O_2 concentration, labile C and NO_3^- availability (Philippot *et al.*, 2007). Thus, under flooding conditions, the rate of denitrification can be directly altered through plant O_2 and C supply, and competition for N between plants and denitrifiers (Engelaar *et al.*, 1995, Whipps, 1984, Engelaar *et al.*, 1991). On the other hand, denitrification rate may be indirectly changed via re-structuring the denitrifying communities in response to flooding, thereby altering physiological properties which drive denitrification kinetics (Schimel & Gulledge, 1998). However, the response of denitrifying communities to environmental changes, particularly flooding in agro-ecosystems, remains largely unknown.

Crop fields subjected to flooding-drying cycles are at risk of significant amounts of N loss due to nitrification followed by denitrification, thereby negatively influencing crop productivity (Burford & Bremner, 1975). N supply may alleviate plant stress due to flooding (Huang *et al.*, 1994, Hamonts *et al.*, 2013). It suggests that in nutrient-limited ecosystems, the magnitude of the denitrification response to flooding may be large due to strong competition between plants and microorganisms for N when compared to high yielding cropping systems with the optimized N fertilization.

Overall, nitrification and denitrification often occur simultaneously when crops suffer flooding periods, potentially leading to severe N limitations in soils. Consequently, crop productivity is likely to be significantly reduced in the absence of effective management practices. More importantly, the impact of flooding on nitrification-denitrification may establish a legacy effect on soil conditions in subsequent growing seasons. Thus, a complete mechanistic understanding of nitrification and denitrification in response to

flooding will help develop effective N managements to minimize N loss from the plant-soil system and avoid any long-lasting, negative effects.

1.3.4.2 Response of soil nitrification and denitrification to drought

Drought events result in strong soil water imbalances which may significantly affect N transformations, particularly nitrification (Fuchslueger *et al.*, 2014). Droughts often reduce the rate of nitrification due to osmotic regulation, lower diffusion rates of substrates and extracellular enzymes, and decreased microbial activities and mobility (Stark & Firestone, 1995, Voroney, 2007). Additionally, under drought conditions, plant root biomass and exudates may decrease (Brunner *et al.*, 2015), thereby possibly influencing the activity of nitrifiers. However, stimulated nitrification in response to drought events may be feasible due to increased soil O_2 levels and decreased plant competition for N due to inhibited plant performance (Kowalchuk & Stephen, 2001, Fromin *et al.*, 2010). Thus, the magnitude and direction of the nitrification response to drought depend not only on the duration and severity of drought periods, but also other factors such as soil type, sampling dates and climatic conditions of sites.

It has been suggested that alterations in nitrification rates in response to drought events may be the consequence of drought-induced changes in ammonia-oxidizing community structure (Pesaro *et al.*, 2004). Previous studies have reported contradictory results in the response of nitrifying communities to drought periods (Hartmann *et al.*, 2013, Fuchslueger *et al.*, 2014, Szukics *et al.*, 2010, Gschwendtner *et al.*, 2014). For example, Fuchslueger *et al.* (2014) observed significantly reduced nitrification rates of mountain grasslands under drought conditions, but no changes in ammonia-oxidizing community structure were observed. In contrast, a decrease in nitrification rate and shifts in nitrifying community structure when soil was dried to 30% of water-filled pore space (WFPS) were apparent in a pristine forest study by Szukics *et al.* (2010). Such inconsistent evidence suggests the nitrifying community response to drought events may be ecosystem-dependent and still poorly understood (Gschwendtner *et al.*, 2014).

Drought periods followed by heavy rainfall generally generate a pulse of C and N fluxes due to microbial resuscitation, and hence results in peaks of nitrification (Placella & Firestone, 2013, Fierer & Schimel, 2002). Significant increases in nitrification after re-wetting dry soils not only contributes to an increase in plant-available N sources, but is also the prerequisite for N loss from the plant-soil system (Bender *et al.*, 2015), and hence risks of reduced crop yields (Vagstad *et al.*, 1997). Nitrification drivers, AOA and AOB, commonly cohabit in soils and their ratio (AOA/AOB) does not always equate to functional dominance (Jia & Conrad, 2009, Xia *et al.*, 2011). Little is known about the recovery of nitrifiers after re-wetting dry soils, and the extent to which AOB and AOA are considered as potentially, functionally redundant in response to drying-rewetting cycles although they have been shown to be distinct in cellular, genomic and physiological characteristics (Thion & Prosser, 2014).

Regarding denitrification, the microbial osmolyte production requirement and the reduction in soil moisture, substrate and extracellular enzyme diffusion, microbial mobility, and plant performance due to drought conditions are expected to decrease denitrification activity (Stark & Firestone, 1995, Roeβler & Müller, 2002, Gschwendtner *et al.*, 2014). The denitrification rate often negatively responds to drought stress in various ecosystems (Groffman *et al.*, 1991, Davidson *et al.*, 1993, Frank & Groffman, 1998, Van Haren *et al.*, 2005, Guo *et al.*, 2014). However, it has sometimes been reported to be stable under drought stress (Larsen *et al.*, 2011, Hartmann *et al.*, 2013). This was explained by the denitrifying enzyme potential capable of enduring drought, or a quick occurrence of de novo enzyme synthesis, thereby capturing the activity of newly produced enzymes in the assay (Hartmann *et al.*, 2013). Similar to nitrification, denitrification is facilitated after re-wetting dry soils due to a flush of C and N mineralization in addition to reduced O_2 levels (Davidson *et al.*, 1993, Keil *et al.*, 2015, Szukics *et al.*, 2010, Guo *et al.*, 2014), leading to an increase in N loss and potentially negative consequences for crop productivity (Gschwendtner *et al.*, 2014, Vagstad *et al.*, 1997). Alterations in the rate of denitrification in response to dry-rewetting cycles are related to soil moisture, nutrient availability and organic matter, but also may be

attributed to changes in the denitrifying community structure (Pesaro *et al.*, 2004, Fierer & Schimel, 2003). A limited number of studies on microbial population dynamics in response to drying-rewetting cycles have provided contradictory results (Szukics *et al.*, 2010, Stres *et al.*, 2008, Hartmann *et al.*, 2013, Palmer *et al.*, 2016). For example, Szukics *et al.* (2010) observed pronounced shifts in the denitrifying community structure under drying-rewetting conditions in forest soils, whereas the denitrifying community persisted through drought periods in a grassland soil study by Hartmann *et al.* (2013). Such contrasting data implies ecosystem-dependent microbial community responses to water deficit stress. Thus, the response of denitrification to extreme drought-rewetting cycles, especially in agricultural systems remains largely unknown.

In general, drought periods often decrease microbial activities, particularly nitrification and denitrification in soils mainly due to limitations in soil water availability. Given that these processes are microbially-driven, little is known about the role of the nitrifying and denitrifying communities in driving the process rate response to drought stress. Importantly, extreme drought events may establish a legacy effect on subsequent growing seasons. Thus, a complete understanding of how two key N processes, nitrification and denitrification, respond to dry-rewetting cycles will help to develop effective N management strategies for sustainable agricultural production under future global changes.

1.4 Knowledge gaps

The Earth's climate system is changing, particularly with a continuing increase in the atmospheric CO_2 levels and increasing global temperatures by the end of 21st century (IPCC, 2013). Simultaneously, the frequency and intensity of extreme weather events are also predicted to increase (IPCC, 2013). Agriculture is known to be highly sensitive to climate variations, and hence sustaining crop productivity is crucial for ensuring the global food and fiber security under future climate conditions (Howden *et al.*, 2007, Tilman *et al.*, 2002, Kang *et al.*, 2009). Soils play a key role in agricultural production due to its involvement in driving nutrient cycling for plant acquisition (Krishna, 2002). The N

cycle is driven by microorganisms, which determines the availability of N in soils, and subsequent consequences for crop growth and productivity (Nelson *et al.*, 2016, Hauck, 1984). Therefore, changes in soil N cycling due to global changes are expected to have profound effects on crop yields (Brevik, 2010).

To date, there have been a large number of studies addressing how plant productivity responds to global changes through focusing on plant physiology and soil physiochemical dynamics across various ecosystems such as humid tropical, temperate and polar regions (Tissue *et al.*, 1993, Nash & Graves, 1993, Lamb *et al.*, 2011, Brown *et al.*, 2012, Rütting *et al.*, 2010, Hatfield & Prueger, 2015). In agro-ecosystems, CO_2 elevation often has positive impacts on crop productivity (Reddy *et al.*, 1997, Sun *et al.*, 2012) whereas the response of crop yields to climate warming depends on crop type and the magnitude of temperature (Lobell & Gourdji, 2012). Regarding extreme weather events including prolonged-drought and flooding, crop productivity responses to these factors are often negative (Bange *et al.*, 2004, Wang *et al.*, 2016). Although soil nutrient availability, particularly N, is regulated by soil microbially-driven processes (Singh *et al.*, 2010, Nelson *et al.*, 2016), less effort has been devoted to discerning how soil microorganisms respond to global changes (Brevik, 2012, Hu *et al.*, 2015). Nitrification and denitrification are two key processes of soil N cycling, contributing to plant-available N sources and N loss via gas emission and leaching, respectively (Ward *et al.*, 2011, Freney & Simpson, 2013). However, little is known about the responses of nitrification and denitrification to global changes and subsequent consequences for crop productivity in agricultural systems.

In order to develop robust and effective predictive models and adaptation strategies for N transformations and relative crop productivity that contribute to support sustainable agricultural production under future climatic conditions, more studies are needed to address these key knowledge gaps:

1. The knowledge of crop productivity and how soil N processes respond to climate change based on single factor models may be limited since little is known about the interaction of elevated CO_2 and temperature, particularly whether combined

impacts are additive, synergistic, or antagonistic compared to the sum of each single effect.

2. Given nitrification and denitrification are microbially-driven processes, the role of nitrifying and denitrifying communities in maintaining the rates of these processes, how they respond to environmental changes and consequences for nitrification and denitrification rates remain largely unknown. Thus, more studies are required to elucidate the relationship “process-associated microbial communities”.
3. Soil water availability is a key factor shaping N and C availability and structuring microbial communities and activities in soils. Thus, extreme weather events including prolonged-drought and flooding may establish a legacy effect on N processes and crop yields for subsequent years. However, little is known about the long-lasting effects following prolonged drought and flooding on soil processes and the subsequent consequences for crop productivity.
4. Ammonia-oxidizers, AOB and AOA, cohabit in most soils and increasing evidence shows their distinctness in cellular, genomic, and physiological features, suggesting that they may respond differently to environmental changes. However, the relative contribution of AOB and AOA to the nitrification process remains largely unknown, particularly under extreme weather events.
5. Despite the fact that large amounts of N fertilizers are often used in high yielding cropping systems, the effects of different rates and types of N fertilizer addition on soil microbial communities and subsequent consequences for crop yields remain relatively unknown. A complete understanding of how soil microorganisms respond to external N supplies will help to effectively manage soil N for crop acquisition, especially under future climatic conditions.

1.5 Aims of this study

This PhD research project generally sought to address the role of soil in mediating crop productivity responses to predicted climate change and extreme weather events including pro-longed drought and flooding. Soil N cycling, particularly nitrification and denitrification were the main focus of the thesis. Efforts were made to elucidate the impacts of future climatic conditions and extreme weather events on these soil processes and subsequent consequences for crop yields via an understanding of the responses of microbial communities and their functioning, thereby providing insights into the relationship "process-associated microbial communities". Additionally, the legacy effects of extreme weather events and the impacts of external N supply on soil processes and microbial communities, and subsequent consequences for crop productivity were also examined.

Cotton cropping was used as a model system to investigate the above knowledge gaps. Cotton is often grown in warm regions and strongly influenced by temperature to sustain crop yields (Wanjura *et al.*, 1969). Cotton is highly sensitive to future climatic conditions and extreme weather events (Bange *et al.*, 2016). Cotton also strongly depends on large amounts of N fertilizer to maintain high crop productivity (Devlin & Chang, 2015). A deep understanding of soil N cycling responses to global changes, the recovery of soils after extreme weather events, and subsequent consequences for cotton crop productivity will support the development of robust predictive models and adaptation strategies for effective managements of N to maintain high cotton crop yields under future climatic conditions.

1.6 Thesis outline

To address how soil N cycling responds to predicted climate change and extreme weather events, the recovery of soils after exposure to extreme weather events, and subsequent consequences for crop productivity in modern cotton farming systems, my research had the following key objectives:

1. Identification of soil nitrification responses, including the nitrification rate and nitrifying community abundance and structure, to waterlogging, elevated CO_2 and temperature, and consequently the implications for cotton crop productivity (**Chapter 2 and 3**).
2. Identification of the recovery of soils previously exposed to extreme weather events: prolonged-drought and flooding, particularly the role of N fertilizer supply in mediating soil process and microbial community performances. The rate of soil N mineralization, nitrification, and denitrification as well as nitrifying and denitrifying communities, and cotton crop productivity in response to varied rates of N fertilizer supply were examined (**Chapter 4**).
3. Identification of the total soil bacterial community and the rate of soil microbial respiration to legacy effects of extreme weather events and different rates of N fertilization (**Chapter 5**).

To accomplish these above objectives, the manipulative field experiments were undertaken at the Australian Cotton Research Institute (ACRI) at Narrabri, NSW. Controlled laboratory approaches using soil physicochemical and process rate analyses, and molecular microbiology were performed at the Hawkesbury Institute for the Environment (HIE), Western Sydney University (WSU), NSW.

Chapter 2 examined the impacts of waterlogging on soil nitrification rate and ammonia-oxidizing communities, and consequently implications for cotton crop productivity. This experiment was conducted by simulating waterlogging events for 120 hrs at either the early and late flowering stages of cotton in the field at ACRI, NSW.

Chapter 3 examined the impacts of elevated CO_2 and temperature on soil nitrification and ammonia-oxidizing communities, and consequently provided implications for cotton crop productivity by using field-based environmentally-controlled chambers to simulate elevated CO_2 (550 ppm) and elevated temperature (+2-4^oC) in the cotton field at ACRI, NSW.

Chapter 4 examined the recovery of soils after exposure to extreme weather events: prolonged-drought and flooding, using varied rates of N-fertilizer addition to elucidate the performances of soil N mineralization, nitrification, and denitrification as well as ammonia-oxidizing communities and denitrifying communities, and consequently cotton crop productivity. This experiment was conducted in temperature and humidity-controlled glasshouses at WSU, NSW.

Chapter 5 presented the responses of the total soil bacterial community and soil respiration to varied N application rates for soils after exposure to extreme weather events: prolonged-drought and flooding. These results were achieved from the glasshouse experiment mentioned in Chapter 4.

Chapter 6 summarized the most important findings and indicated future perspectives of this PhD research project.

CHAPTER 2 IMPACTS OF WATERLOGGING ON SOIL NITRIFICATION AND AMMONIA-OXIDIZING COMMUNITIES IN COTTON FARMING

2.1 Introduction

Soil nitrification plays an important role in providing NO_3^- for plant uptake, and potentially contributes to N loss through NO_3^- leaching and N_2O emissions (Hirsch & Mauchline, 2015). Therefore, alterations in factors controlling process efficiency are likely to influence soil N availability and ultimately crop productivity. Nitrification requires aerobic conditions and consists of two sequential steps: (1) the rate-limiting ammonia oxidation to convert NH_3 to NO_2^- via hydroxylamine by ammonia-oxidizing microorganisms, and (2) nitrite oxidation to produce NO_3^- by nitrite-oxidizing bacteria (Bernhard, 2012). It has been long known that ammonia-oxidizer communities are mainly composed of β- and γ-*Proteobacteria*; however, isolation of archaea belonging to the phylum Thaumarchaeota capable of oxidizing NH_3 has changed this perception (Könneke *et al.*, 2005). AOA are generally abundant in soils and outnumber their counterpart AOB (Leininger *et al.*, 2006, Nicol *et al.*, 2008). The coexistence of AOB and AOA in soils has led to an increasing number of research studies investigating their relative contribution to nitrification, which is still not fully understood.

Cotton, a valuable industrial crop contributing to world fiber production, is immensely dependent on N supply to maintain high productivity. The application of N fertilizers ranging from 240-270 kg N ha^{-1} has been estimated to optimize the productivity of Australian cotton crops (Rochester & Constable, 2015). Previous studies have reported that approximately 32% of crop N was derived from N fertilizers (Rochester *et al.*, 1993, Rochester *et al.*, 1994). In the context of increasing frequency and intensity of extreme weather events such as flooding, the cotton industry is expected to face the risk of reduced crop productivity as a consequence of soil N depletion and altered plant physiological processes, including reductions in photosynthesis, transpiration and

radiation-use efficiency (Sahay, 1989, Bange *et al.*, 2004). Upland cotton plants (*Gossypium hirsutum* L.) are mainly grown on heavy clay soils (vertosol) with very low rates of drainage (Hodgson & Chan, 1982) under furrow irrigation systems in Australia. Such conditions may promote waterlogging events in the case of poor irrigation management following substantial precipitation (Bange *et al.*, 2004). The Australian cotton industry suffered a loss of approximate A$300 million in 2011 as a consequence of yield damage caused by waterlogging and considerable heavy rainfall (CRC, 2010-2011)

Waterlogging alters soil O_2 concentrations, which is a factor controlling the nitrification process. In particular, excessive water content in the soil upon waterlogging decreases O_2 diffusion capacity, leading to hypoxic or even anoxic environments that inhibit the activity of nitrifying communities, resulting in depleted soil N availability that will negatively affect N-dependent cotton crop productivity. The rate of nitrification is expected to decrease in response to waterlogging conditions (Reddy & Patrick, 1975); however, the underlying mechanism remains largely unknown. Limited studies have provided contradictory evidence on the impact of waterlogging on nitrification. For example, waterlogging reduced the nitrification rate and did not recover in 4 months (Van Schreven & Sieben, 1972) while another study suggested that nitrification rates remained similar in control and waterlogged soils due to the oxygen supply from the roots of plants (Engelaar *et al.*, 1995). Rates of soil nitrification can be reduced directly by waterlogging due to the reduced availability of oxygen or from changes in substrate availability (Reddy & Patrick, 1975). Alternatively, waterlogging can shift the abundance and/or the surrounding soil structure of ammonia - oxidizing communities, which in turn will impact the rate of nitrification (Gleeson *et al.*, 2010). Despite the fact that nitrification is a microbially-regulated process, only a few studies have examined the role of nitrifying communities in maintaining nitrification rates and how they respond to environmental changes (Hu *et al.*, 2015). In particular, whether and how ammonia-oxidizing communities respond to waterlogging stress remains unclear.

Therefore, in this study, cotton was used as a model crop to explore the response of ammonia-oxidizing communities and nitrification rates to waterlogging. Data obtained from this research will help clarify the links between nitrifying communities and their function. In terms of the agro-economy, the outcomes of this study will contribute towards building knowledge for developing effective strategies for N management for the cotton industry in the face of current climate variability and future global changes, including higher intensity and frequency of flooding. In addressing the aims, I hypothesized that (i) waterlogging will alter nitrification rate and ammonia-oxidizing community due to changes in soil physicochemical properties; and (ii) AOB and AOA will respond differently to waterlogging due to their differences in physiology and ecological niches.

2.2 Materials and Methods

2.2.1 Field site

This field experiment was conducted at the Australian Cotton Research Institute (ACRI) at Narrabri (30.31^{o}S, 149.78^{o}E) in north-west New South Wales, Australia. This region represents a semiarid ecosystem, and experiences hot summers with maximum and minimum daily temperature of 35^{o}C and 18^{o}C, respectively. Annual rainfall is about 644 mm, of which approximately one-third falls during the summer months (Bureau of Meteorology, NSW). The soil is cracking grey clay soil (vertosols) with an alkaline pH of 7.5-8.0; soils are designated as Ug 5.25 under the Northcote classification system (Northcote *et al.*, 1975).

2.2.2 Cotton cultivation

The CSIRO cultivar Sicot 71BRF was used in this experiment. Cotton was planted on ridges spaced 1 meter apart separated by a furrow in which water was applied as furrow-flood irrigation. There were 4 irrigation events in total and each irrigation event provided approximately 90-100 mm of water to follow normal agronomic practices. N was applied as anhydrous ammonia before sowing at a rate of 180 kg N/ha (Braunack, 2013).

Phosphorus and potassium fertilizers were also applied prior to sowing at the rates of 19 kg P/ha and 13 kg K/ha, respectively (Braunack, 2013).

2.2.3 Experimental design

The experimental field was divided into 4 blocks, with each block consisting of three plots. Each plot was 160 meters long and contained 8 rows; each row was 1 meter wide (**Figure 2.1**). Waterlogging was simulated by running furrow irrigation for 120 hours across two different time points. The first waterlogging event (WL1) was applied when cotton plants were in the early flowering stage, whereas the second waterlogging event (WL2) was applied at the final stages of flowering. The flowering stage is considered the most vulnerable period in cotton growth because there is a rapid increase in water and nutrient requirements (Kerby *et al.*, 2010). Waterlogging at two crop developmental stages was undertaken to determine the impact of substrate (NH_4^+) availability on the nitrification process rate; at the final stages of flowering, the soil was anticipated to have significantly lower NH_4^+ due to nitrification and plant uptake. WL1 began on 16 January 2014 and ended 21 January 2014, and WL2 began on 7 February 2014 and ended 11 February 2014. During the experiment, there were 4 regular furrow irrigation events and each ran for 8 hours; irrigations were applied on 16 and 29 January, and 7 and 21 February, 2014.

Field lay-out of plots and treatments:

Block 1			Block 2			Block 3			Block 4		
Plot 1 +WL2	Plot 2 +WL1	Plot 3 Control	Plot 4 Control	Plot 5 +WL2	Plot 6 +WL1	Plot 7 +WL1	Plot 8 +WL2	Plot 9 Control	Plot 10 Control	Plot 11 +WL1	Plot 12 +WL2

Treatment 1= Control

Treatment 2= +WL1

Treatment 3= +WL2

Figure 2.1 Detailed field-based experimental design: The field layout of treatments at ACRI in Narrabri, NSW. There were 4 blocks (replications) consisting of 12 different plots in total. The waterlogging treatments were randomly applied in plots. The first waterlogging events occurred at the early flowering stage, and the second waterlogging events occurred at the late flowering stage. WL1 = Waterlogging 1; WL2 = Waterlogging

Figure 2.2 Photo of cotton field at ACRI in Narrabri, NSW when soil was subjected to the first waterlogging event (WL1) at the early flowering stage by running furrow irrigation for 120 hrs. WL1 = Waterlogging 1; WL2 = Waterlogging 2.

2.2.4 Soil sampling

Soil cores of 4 cm diameter and 10 cm deep were taken from the waterlogged fields before and 4, 16, 22, 28, and 51 days after the first waterlogging event; before and 5 and 28 days after the second waterlogging event. Soil cores were also collected from control plots 3 days after the first irrigation event and at days which were corresponding to the sampling time points of samples collected from WL1 and WL2 plots. During the experiment, furrow irrigation was applied four times: (1) the first irrigation (120 hrs to simulate WL1; 8 hrs for control and WL2 plots); (2) the second irrigation at day 11 after WL1 (8 hrs for all plots); (3) the third irrigation at day 21 after WL1 (120 hrs to simulate WL2; 8 hrs for WL1 and control plots), and (4) the last irrigation 17 days before the end of the experiment (8 hrs for all plots). Soil samples were then transferred to the Hawkesbury Institute for the Environment (HIE), Western Sydney University for further analysis. At HIE, soil samples were homogenized and sieved through 4 mm mesh, and then stored at 4^{o}C for chemical analyses. Subsamples of soil were kept at -20^{o}C for molecular analyses.

2.2.5 Soil physicochemical analyses

Soil gravimetric water content was determined by drying 3 grams of fresh soil in the oven at 105^{o}C for 24 hrs. For soil pH, a suspension of fresh soil and milli-Q water (in a ratio of 1:5) was made and shaken for 1 hr, prior to being measured with a pH meter (Seve-nEasy pH, Metler, Toledo, Switzerland). Soil NH_4^+ and NO_3^- were measured by taking 5 g of fresh soil samples and then mixed with 50 mL of 2M KCl (Keeney & Nelson, 1982). The mixture was shaken at 180 rpm for 1 hr, and then filtered by Whatman no. 42 filter paper before the filtrate was collected. The NH_4^+ and NO_3^-concentrations of the filtered solutions were analyzed by a SEAL AQ2 discrete analyzer (SEAL analytical Inc., USA). Soil total N and C were determined by taking fresh soils that were dried at 40^{o}C for at least 72 hrs, and then ground to a fine powder. Soils were analyzed a LECO macro - CN analyzer (LECO, USA) to determine the C and N concentration.

2.2.6 Potential nitrification rate

The potential nitrification rate was determined according to the chlorate inhibition method (Kandeler & Böhm, 1996). Five grams of fresh sieved soil was placed into 125 mL bottle and 20 mL of 1mM PBS containing 1 mM $(NH_4)_2SO_4$ and 50 mgL^{-1} of $KClO_3$ was added to each sample. The sample then was incubated on a shaker at 150 rpm, 25^oC for 5 hrs. For the control, the same amount of sieved soil was placed into a similar bottle and 20 mL of 1 mM PBS containing 1 mM $(NH_4)_2SO_4$ was added and then frozen at -20^oC for 5 hrs. After incubation, the control was thawed to RT. All samples had 5 mL of 2 M KCl added, and then were securely placed on a shaker for 10 min at 150 rpm. Finally, samples were filtered immediately through Whatman no. 42 filter paper. Two color reagents including sulfonic acid ($NH_2C_6H_4SO_3H$) dissolved in 12% acetic acid (CH_3COOH) and naphthylamine dissolved in 20% acetic acid (CH_3COOH) were in turn added into the extracts (Liu *et al.*, 2016, Hu *et al.*, 2015). Subsequently, 200 µl of each solution was pipetted into a microplate, and then the absorbance at 520 nm wavelength was measured by the spectrophotometer microplate reader (Enspire® Multilable Reader, Perkin, Elmer, USA). Absorbance values were converted into N concentrations using a standard curve formulated from a series of $NaNO_2$ concentrations.

2.2.7 Microbial community analyses

2.2.7.1 DNA extraction

Total genomic DNA was extracted from 0.25 g of soil using the MoBio PowerSoil DNA Isolation kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instruction, using a FastPrep bead beating system (Bio-101, Vista, CA, USA) at a speed of 5.5 m s^{-1}. The quantity and quality of extracted DNA were checked photometrically using a NanoDrop® ND-2000c UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Extracted DNA was kept at -20^oC until further analysis.

2.2.7.2 Quantitative PCR

The bacterial and archaeal *amoA* gene (AOB and AOA *amoA*) abundances were quantified using the BioRad C1000 Touch thermal cycler CFX96 Real-Time system (Bio-Rad laboratory, USA), using two pairs of primers: CrenamoA23f/CrenamoA616r (ATGGTCTGGCTWAGACG/GCCATCCATATGTATGTCCA) (Tourna *et al.*, 2008) and amoA-1F/amoA-2R (GGGGTTTCTACTGGTGGT/CCCCTCKGSAAAGCCTTCTTC) (Rotthauwe *et al.*, 1997) for AOA and AOB *amoA* genes, respectively. Each sample was quantified in a 10 µl reaction including 5 µl GoTaq® qPCR Master Mix (2X), 20µM each primer, 0.1 µl CXR reference dye and 10 ng of template DNA. The PCR thermal conditions for AOB *amoA* gene amplification were as follows: an initial cycle of 95^{o}C for 10 min; 39 cycles of 94^{o}C for 45 s, 58^{o}C for 45 s, to 72^{o}C for 45 s; 1 cycle of 95^{o}C for 15 s, 60^{o}C for 30 s, to 95^{o}c for 15s (Hallin *et al.*, 2009). The PCR thermal conditions to amplify AOA *amoA* gene were: an initial cycle of 95^{o}C for 10 min; 39 cycles of 94^{o}C for 45 s, 55^{o}C for 45 s, to 72^{o}C for 45 s; 1 cycle of 95^{o}C for 15 s, 60^{o}C for 30 s, to 95^{o}c for 15 s (Hallin *et al.*, 2009).

Standards for qPCR were constructed by cloning isolated AOA and AOB *amoA* genes into the pCR®4–TOPO vector (Invitrogen, Carlsbad, CA). A 10-fold serial dilution of plasmid was prepared to generate standard curves. Melt curve analyses were conducted following each assay to verify the specificity of the amplification products. PCR efficiencies for different assays ranged between 86% and 97%.

2.2.7.3 Terminal restriction fragment length polymorphism

The structure of AOA and AOB communities was determined using TRFLP. AOB and AOA *amoA* gene fragments were amplified using fluorescently labelled primers FAM-CrenamoA23f/CrenamoA616r and VIC-amoA-1F/amoA-2R respectively. PCR was conducted using a 25 µl reaction volume including 2.5 µl of 10 x NH_4 reaction buffer (10x, Bioline, Australia), 0.5 µl of 20 mM deoxynucleoside triphosphate (dNTP mix, Bioline, Australia), 0.25 µl of each primer with the concentration of 20 µM (Sigma Aldrich, Australia), 1 µl of BSA (20 mg/ml, NewEngland Biolabs, USA), 1 µl of 50 mM $MgCl_2$

(Bioline, Australia), 0.25 µl Taq DNA polymerase (Bioline, Australia) and 10 ng of DNA template. After denaturing the PCR mixture at 95^{o}C for 5 min, DNA template was amplified with 35 cycles of denaturing at 95^{o}C for 30 s, annealing at 56^{o}C for 30 s, and extension at 72^{o}C for 1 min, and finally extension at 72^{o}C for 10 min on a Dyad Peltier Thermal cycler (Biorad, Australia). PCR amplicons were then visualized on 1% (w/v) agarose gel under UV radiation to check for successful amplification.

Quadruplicate PCR products from each sample were pooled, and then purified using the Wizard SV Gel and PCR clean-up System (Promega, San Luis Obispo, CA, USA) according to manufacturer's instructions. The concentration of purified PCR products was measured by NanoDrop® ND-2000c UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The concentration of purified DNA ranged from 40 to 100 ng/µl. The ratio of $A_{260/280}$ and $A_{260/230}$ varied in the range of 1.8-2.1 and 0.7-1.7, respectively.

Purified PCR products were then subjected to digestion using commercial restriction enzymes. A 10 µl reaction volume composed of 200 ng of DNA, 1 µl of 10x NEB buffer, 0.1 µl of BSA and 5U of restriction enzyme. Two types of restriction enzymes including MspI and HpyCH4V (New England BioLabs, USA) were used for AOB and AOA, respectively. These enzymes were used because previous studies reported that they provide high discrimination between samples (Yao *et al.*, 2011, Hu *et al.*, 2015). Digests were incubated at 37^{o}C for 3 hrs, followed by 95^{o}C for 10 min to deactivate the restriction enzymes. Terminal restriction fragments (TRFs) were resolved on an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, CA, USA). A geneScan 600-LIZ internal size standard (Applied Biosystems) was applied to each sample. Genemapper version 4.0 (Applied Biosystems) was used to analyze the TRFLP profile. The output from Genemapper was imported into T-REX software for further analysis (http://.terex.biohpc.org; Culman *et al*., 2009), including quality control procedures by terminal restriction fragment (TRF) alignment (clustering threshold = 0.9 pb), and noise filtering (peak area, standard deviation multiplier = 1). The relative abundance of TRFs

was calculated based on peak height. TRFs with peak heights comprising less than 2% of the total peak height were removed from downstream analyses to avoid artifacts (Hu *et al.*, 2015).

2.2.8 Statistical analysis

One-way analysis of variance was conducted to examine the differences in soil physicochemical properties, potential nitrification rate, and the abundance of ammonia-oxidizing communities before and after waterlogging treatments. The *amoA* gene copy numbers were log-transformed prior to statistical analysis to meet normality assumptions. Spearman's rank test was used to evaluate the degree of correlations among soil physicochemical properties, AOB and AOA *amoA* gene copy numbers, AOB and AOA community structures, and potential nitrification rate. One-way ANOVA and Spearman's rank tests were conducted using SPSS 22 (IBM, Armonk, USA).

Non-metric multidimensional scaling (NDMS) was used to visualize the Bray-Curtis dissimilarity matrices based on the relative abundance of AOA and AOB TRFs using Primer v6 (PRIMER-E Ltd, Plymouth, UK). Significance of Bray-Curtis dissimilarity was examined by PERMANOVA. $P<0.05$ was considered to be statistically significant.

2.3 Results

2.3.1 Treatment effects on soil physicochemical properties

2.3.1.1 Soil moisture content

There was a clear difference in soil moisture values after waterlogging treatments were applied to cotton crops. Soil moisture content increased by approximately 10% 4 days and 5 days after WL1 and WL2 treatments when compared to control plots (**Figure 2.3**). Prior to WL1, no significant differences in soil moisture content were observed for soil samples from control plots and WL1 plots ($P=0.878$). Similarly, the moisture content of soil samples from control and WL2 plots before WL2 applied were quite similar ($P=0.089$). After 4 days of WL1 event, there was a significant difference in soil moisture

content between control and the WL1 plots (P=0.001). The moisture content between WL2 and control plots were also significantly different (P<0.001) 5 days after WL2 treatment had been applied. At the end of the experiment, there was no significant difference of soil moisture among different treatments (P=0.165).

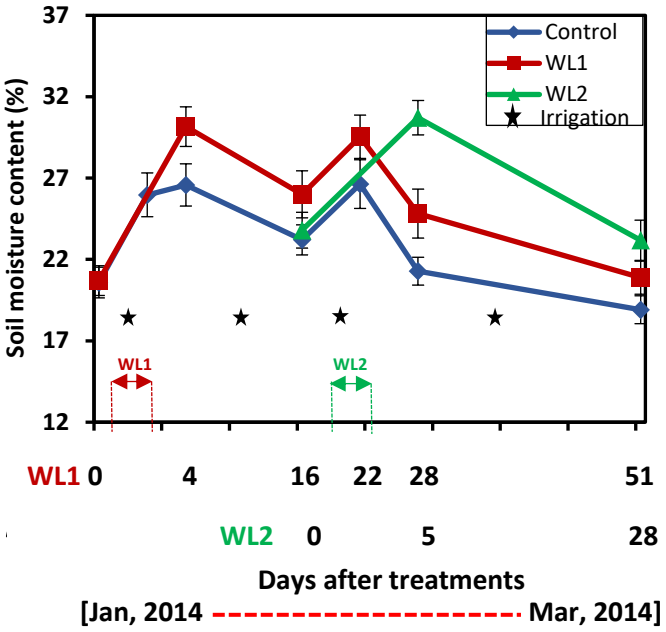

Figure 2.3 Changes in soil moisture before and after waterlogging treatments were applied. The experiment started in January and ended in March, 2014. During the experiment, four furrow irrigation events were applied. Data are presented for days after treatments. The first line along the x-axis indicates days after WL1 was applied. The second line along the x-axis indicates days after WL2 was applied. Values represent mean ± SE (n=12 for control samples at Day 0 and 4 after WL1 and 2 days after the first irrigation event and WL1 samples at Day 0 and 4; and n=8 for the rest of data) of each treatment. WL1 = Waterlogging 1; WL2 = Waterlogging 2.

2.3.1.2 Soil pH

Soil pH considerably increased after waterlogging treatments. For the first waterlogging event, soil pH increased from 7.9 to 8.2 whereas it increased from 7.8 to 8.0 for the second waterlogging event (**Figure 2.4**). Soil pH in control plots slightly increased after

irrigation and then decreased gradually (**Figure 2.4**). Statistical analysis showed that there were significant differences in soil pH between controls and plots with waterlogging treatments applied (P<0.001). At the end of the experiment, there were differences in soil pH among control, WL1 and WL2 treatments. In particular, the pH values observed in the control plot was 7.5 whereas pH values of 7.95 and 7.84 belonged to WL1 and WL2, respectively.

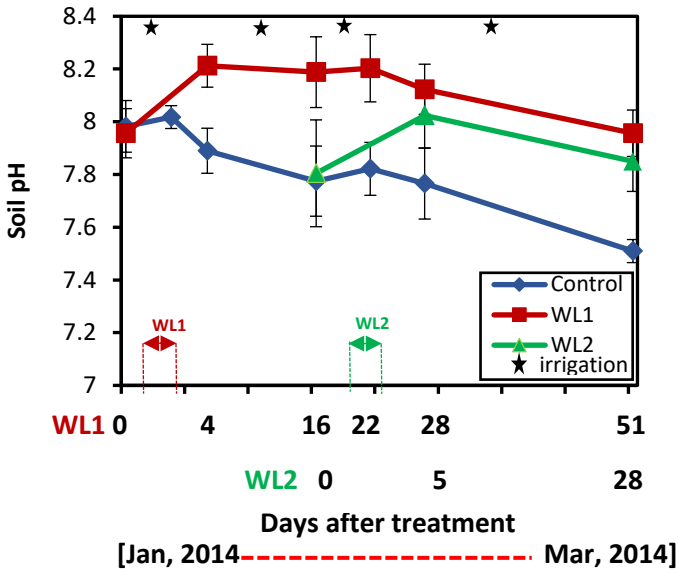

Figure 2.4 Changes in soil pH values before and after waterlogging treatments were applied. The experiment started in January and ended in March, 2014. During the experiment, four furrow irrigation events were applied. Data are presented for days after treatments. The first line along the x-axis indicates days after WL1 was applied. The second line along the x-axis indicates days after WL2 was applied. Values represents mean ± SE (n=12 for control samples at Day 0 and 4 after WL1 and 2 days after the first irrigation event and WL1 samples at Day 0 and 4; and n=8 for the rest of data) of each treatment. WL1 = Waterlogging 1; WL2 = Waterlogging 2.

At the start of the experiment, soil total nitrogen values were 0.86 g/kg dry soil for samples collected from WL1 and control plots. Afterwards, soil N values decreased gradually during the experiment (**Figure 2.5**). Statistical analysis showed a significant

difference in soil total N between WL1 and control plots (P<0.001). In particular, WL1 decreased soil total N by 15%. A similar trend on soil total N was observed for the WL2 treatment, but the difference was not statistically significant (P=0.192).

A downward trend was obtained for the concentration of $NH_4^+$and NO_3^- in the soil samples collected from control and waterlogging treatments (**Figure 2.5**). Before WL1 was applied, the concentration of NH_4^+ was similar for both control and WL1 plots (P= 0.634), whereas it was significantly different (P=0.003) after 3 days of waterlogging treatment. The amount of NH_4^+ decreased by about 9.5% after WL1. However, no significant difference in NH_4^+ concentration was observed either before or after WL2 (P=0.582 and P=0.403). The concentration of NO_3^- before WL1 treatment was not different (P=0.326). After 3 days of treatment, the amount of NO_3 was significantly different between control and WL1 plots (P<0.001). The same result was obtained for the WL2 event. No significant difference in NO_3 concentration was observed before WL2 treatment (P=0.999), however NO_3^- concentrations between control and WL2 were significantly different (P<0.001) after waterlogging. Particularly, soil NO_3^- concentration decreased approximately 40% and 50% for WL1 and WL2, respectively. At the end of the experiment, the amount of inorganic N in the soil had dropped to very low amounts (0.66 mg NH_4^+/kg dry soil and 3.17 mg NO_3^-/kg dry soil).

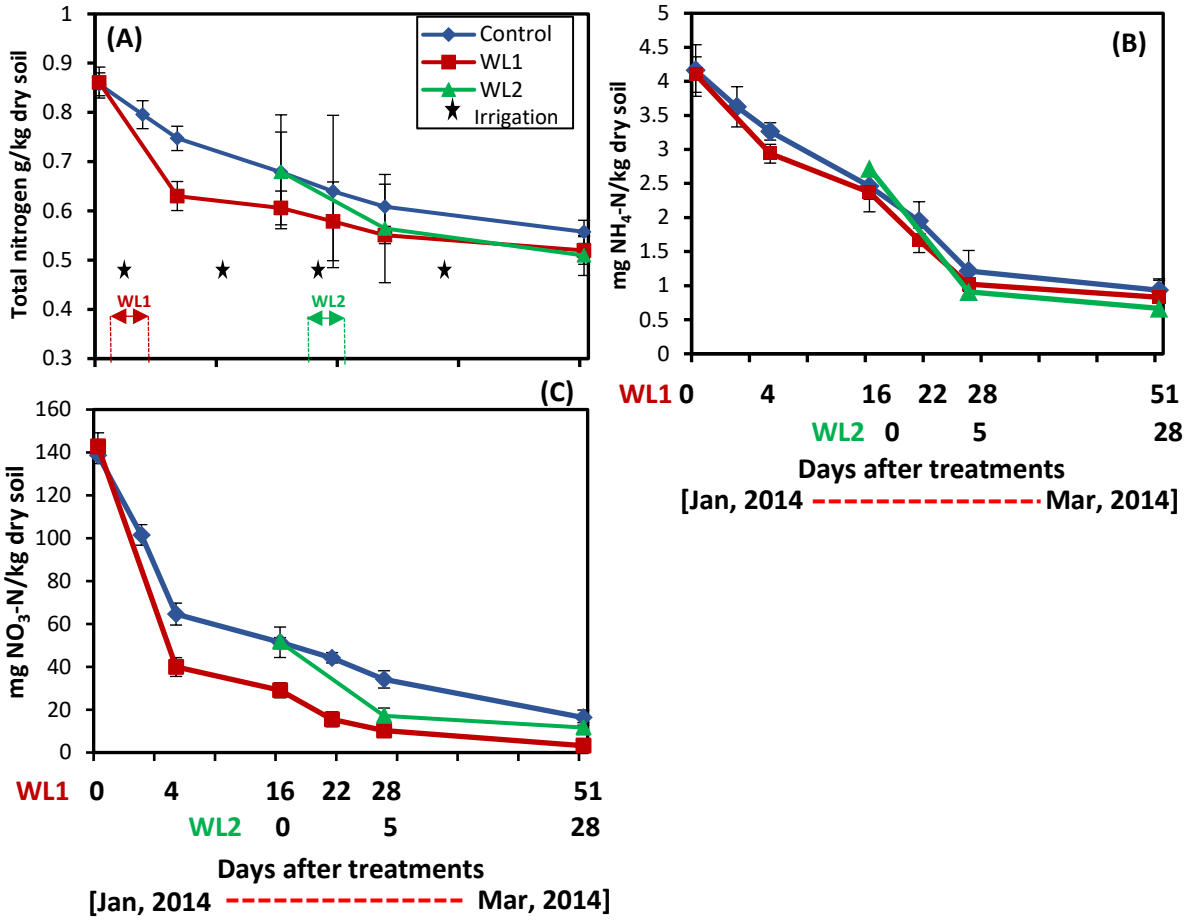

Figure 2.5 Changes in total N and inorganic N before and after waterlogging treatments were applied. The experiment started in January and ended in March, 2014. During the experiment, four furrow irrigation events were applied. Data are presented for days after treatments. The first line along the x-axis indicates days after WL1 was applied. The second line along the x-axis indicates days after WL2 was applied. (**A**) Total nitrogen, (**B**) NH_4^+ concentration, and (**C**) NO_3^- concentration. Values represent mean ± SE (n=12 for control samples at Day 0 and 4 after WL1 and 2 days after the first irrigation event and WL1 samples at Day 0 and 4; and n=8 for the rest of data) of each treatment. NH_4^+ = Ammonium, NO_3^-= Nitrate. WL1 = Waterlogging 1; WL2 = Waterlogging 2.

2.3.2 Treatment effects on potential nitrification rate

Potential nitrification rates (PNR) decreased after waterlogging treatments were applied. PNR reduced from 1.2 to 0.9 mg N/kg dry soil/h after WL1 and from 1.1 to 0.9 mg N/kg dry soil/h after WL2 (**Figure 2.6**). PNR of control and WL1 plots were the same before treatment (P=0.797), whereas they were significantly different 4 days after waterlogging (P=0.041). Before WL2 was applied, no significant difference was found for samples collected from control and WL2 plots (P=0.7171). However, PNR was significantly different 5 days after WL2 was applied (P<0.001). The lower PNR measured and the statistically significant difference in PNR after WL1 and WL2 treatments suggest that waterlogging negatively affects the potential nitrification rate.

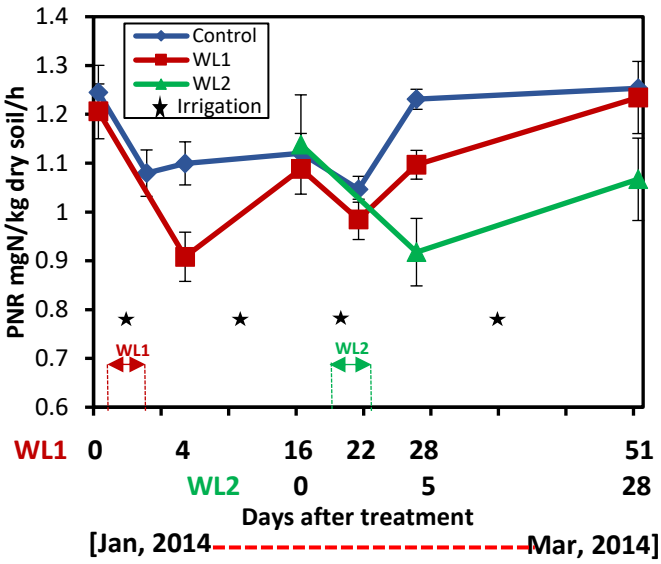

Figure 2.6 Changes in potential nitrification rate (PNR) before and after waterlogging treatments were applied. The experiment started in January and ended in March, 2014. During the experiment, four furrow irrigation events were applied. Data are presented for days after treatments. The first line along the x-axis indicates days after WL1 was applied. The second line along the x-axis indicates days after WL2 was applied. Values represent ± SE (n=12 for control samples at Day 0 and 4 after WL1 and 2 days after the first irrigation event and WL1 samples at Day 0 and 4; and n=8 for the rest of data) of each treatment. WL1 = Waterlogging 1; WL2 = Waterlogging 2.

2.3.3 Treatment effects on ammonia-oxidizer community abundance and structure

2.3.3.1 Ammonia-oxidizer community abundance

AOB *amoA* gene copy number varied from 1.18 x 10^6 to 1.42 x 10^7/g dry soil across all treatments (**Figure 2.7A**). After 4 days of WL1, the AOB *amoA* gene abundance decreased approximately 10-fold. Statistical analysis showed that there were significant differences between the AOB *amoA* gene copy number of control and WL1 plots 4 days after waterlogging (*P*=0.005). Fifteen days after WL1 treatment, the AOB abundance increased approximately 2-fold, and then slightly decreased at Day 22. At Day 28 after WL1, AOB *amoA* gene copy number increased by 52.4% and then marginally changed at Day 51(**Figure 2.7A**). At the end of the experiment (Day 51), the abundance of AOB of WL1 plots was significantly different from that of control (*P*=0.045); however, they differed from each other only 2.5-fold.

The same trend was observed for the WL2 treatment. Five days after WL2, the abundance of AOB dramatically decreased. In particular, AOB abundance of WL2 plots was statistically different from that of control at Day 5 (*P*=0.001). At the end of the experiment which corresponded to 28 days after WL2, no significant difference was found (*P*=0.76).

AOA *amoA* gene copy number ranged from 2.29 x 10^8 to 6.27 x 10^8 /g dry soil across all treatments (**Figure 2.7B**), and AOA outnumbered their counterpart AOB. The AOA *amoA* gene abundance from WL1 and WL2 plots slightly decreased after treatments were applied although there were statistically significant differences between waterlogged and control plots (*P*=0.013 for WL1, and *P*=0.026 for WL2). At the end of the experiment, the abundance of AOA of control plots were approximately 1.4-fold higher than that of WL1 and WL2 plots and they were statistically significant (*P*=0.034 for control and WL1 plots; and *P*=0.041 for control and WL2 plots).

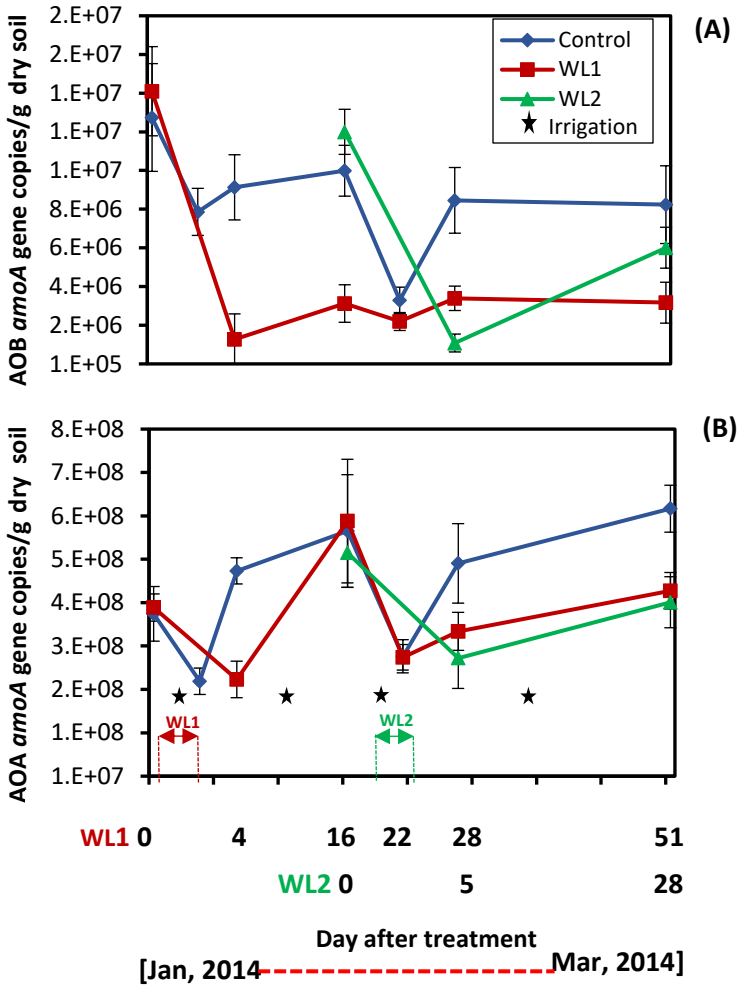

Figure 2.7 Changes in (**A**) AOB and (**B**) AOA *amoA* gene abundance before and after waterlogging treatments were applied. The experiment started in January and ended in March, 2014. During the experiment, four furrow irrigation events were applied. Data are presented for days after treatments. The first line along the x-axis indicates days after WL1 was applied. The second line along the x-axis indicates days after WL2 was applied. Values represent mean ± SE (n=12 for control samples at Day 0 and 4 after WL1 and 2 days after the first irrigation event, and WL1 samples at Day 0 and 4; and n=8 for the rest of data) of each treatment. WL1 = Waterlogging 1; WL2 = Waterlogging 2.

2.3.3.2 Ammonia-oxidizing community structure

The analysis of TRFLP data for the AOB *amoA* gene generated 4 different TRFs for each waterlogging treatment after digestion of PCR products by the restriction enzyme, MspI (**Figure 2.8A**). The AOB community structure changed for samples collected before and after waterlogging. In particular, dominant TRF-55 decreased while TRF-251 increased after WL1. There were statistically significant differences in the relative abundances of TRF-55 and TRF-251 between control and WL1 (P=0.009 and P=0.012, respectively). For the second waterlogging event, dominant TRF-55 also significantly decreased (P=0.007) whereas TRF-251 significantly increased 5 days after the treatment was applied (P=0.011). In contrast to WL treatment, the relative abundance of all TRFs from control plots changed slightly after regular irrigation. The low number of TRFs yielded suggests a low diversity of ammonia-oxidizing bacterial communities in these soils.

TRFLP of AOA *amoA* gene generated 8 different TRFs for all treatments (**Figure 2.8B**). Among these, TRF-54, TRF-74 and TRF-251 were the three most dominant genotypes. AOA community structures changed after waterlogging treatments were applied. There was an increase in the relative abundance of TRF-54 after WL1 and WL2 events; however, no significant statistical differences were observed (P=0.13 and P=0.064, respectively). TRF-74 decreased upon waterlogging treatments and was significantly different from the control (P=0.023 for WL1 and P=0.04 for WL2). The AOA community structures of control plots slightly changed after each regular irrigation before stabilizing.

Both TRFLP and qPCR data showed a decline in the relative abundances of some bacterial and archaeal genotypes upon waterlogging. In TRFLP profiles, TRF-55 and TRF-74 significantly decreased after treatments for AOB and AOA, respectively. In agreement with the TRFLP fingerprint, the *amoA* gene abundance also significantly decreased for AOB and AOA. However, the bacterial *amoA* gene abundance decreased more than that of archaea.

Phylogenetic affiliation of some AOB and AOA *amoA* gene TRFs in my study could be identified according to the field study by Hu *et al*., (2015), in which the phylogenetic affiliation of 11 distinct AOA TRFs and 6 AOB TRFs generated from TRFLP analysis of irrigated and fertilized sandy loam soils planted with *Eucalyptus saligna* were reported. By searching for the identical TRFs from the study by Hu *et al.* (2015), the phylogenetic affiliation of total 7 different TRFs of AOB and AOA in my study could be assigned. In particular, AOB TRF-149 and 229 was placed within the *Nitrosospira* cluster. In terms of the AOA community, TRF-74, 150 and 212 belonged to the *Nitrososphaera* cluster whereas TRF-54 and 198 were associated with the *Nitrosopumilus* and *Nitrosotalea* clusters, respectively (**Table 2.1**).

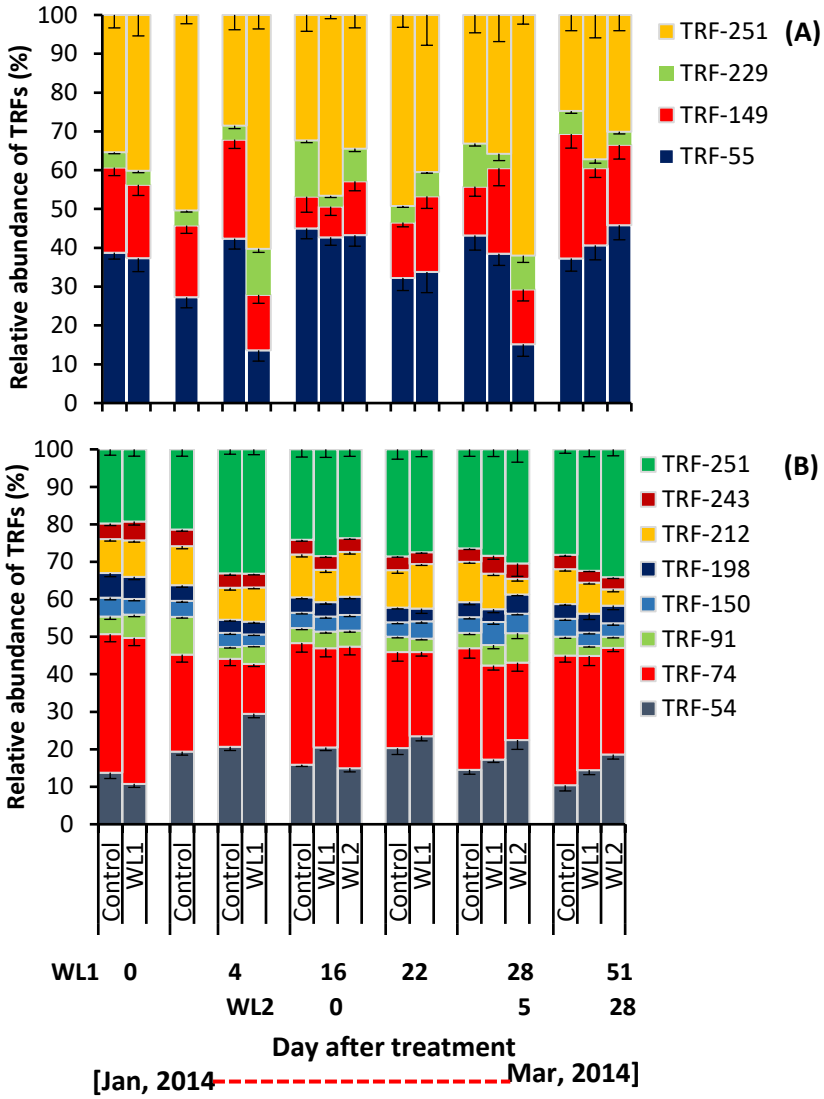

Figure 2.8 TRFLP finger printing of *amoA* gene TRFs before and after waterlogging treatments were applied. The experiment started in January and ended in March, 2014. During the experiment, four furrow irrigation events were applied. Data are presented for days after treatments. The first line along the x-axis indicates days after WL1 was applied. The second line along the x-axis indicates days after WL2 was applied. Values represent ± SE (n=12 for control samples before and 2 and 6 days after the first irrigation event and WL1 samples at Day 0 and 4; and n=8 for the rest of data) of each treatment. WL1 = Waterlogging 1; WL2 = Waterlogging 2.

Table 2.1 Phylogenetic affiliation of (a) AOB and (B) AOA TRFs digested by MspI and HpyCH4V enzymes, respectively

	TRFs	Phylogenetic affiliation (Cluster level)	Reference
(a) AOB	TRF-149	*Nitrosospira*	Hu *et al*., 2015
	TRF-229	*Nitrosospira*	
(b) AOA	TRF-54	*Nitrosopumilus*	Hu *et al*., 2015
	TRF-74	*Nitrososphaera*	
	TRF-150	*Nitrososphaera*	
	TRF-198	*Nitrosotalea*	
	TRF-212	*Nitrososphaera*	

The ammonia-oxidizing community structure including ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) were analyzed by principal coordinates analysis (PCO) derived from the Bray-Curtis dissimilarity matrices for the relative abundance of TRFs. For control and WL1 plots, two first PCOs of AOB community structure explained 95.2% of the variation in the data. Before WL1 treatment was applied, the community structures of control and WL1 were not markedly separated from each other. However, they were distinct after 4 days of the waterlogging treatment being applied (**Figure 2.9A**). For control and WL2 plots, two first PCOs of AOB community structure explained 99% of the variation in the data. The same trend was obtained for WL2. In particular, the AOB community structures of WL2 and control were distinct 5 days after waterlogging treatment (**Figure 2.9B).**

The AOA community structure analysis for control and WL1 plots by two first PCOs explained 72.2% of AOA variation in the TRFLP and clearly showed a separation between control and WL1 samples after 4 days of the waterlogging treatment being applied (**Figure 2.10A**). For control and WL2 plots, two first PCOs of AOB community structure explained 90.2% of the variation in the AOB community. There was also a distinction within the AOA community structure between control and WL2 5 days after waterlogging treatment (**Figure 2.10B**).

Before waterlogging treatments were applied (Day 0), the ammonia-oxidizer communities were expected to be similar between control and WL1; control and WL2; however, PERMANOVA test showed $P<0.05$ (**Table 2.2 & Table 2.3**). Although statistically significant differences in the ammonia-oxidizing community structure between control and WL1; and control and WL2 at Day 0 were obtained, the similarity of AOB, AOA structure between WL1 and WL2 and control plots at Day 0 can be acceptable since PCO ordination showed very close distances (**Figures 2.9 & 2.10**). The ordination plots also showed big changes in AOB and AOA community compositions after 4 and 5 following days of WL1 and WL2, respectively. At Day 16, 22, 28 and 51 after WL1, and Day 5 and 28 after WL2, the AOB and AOA communities showed their gradual resilience by slowly returning to the control positions on each ordination plot (**Figures 2.9 & 2.10**).

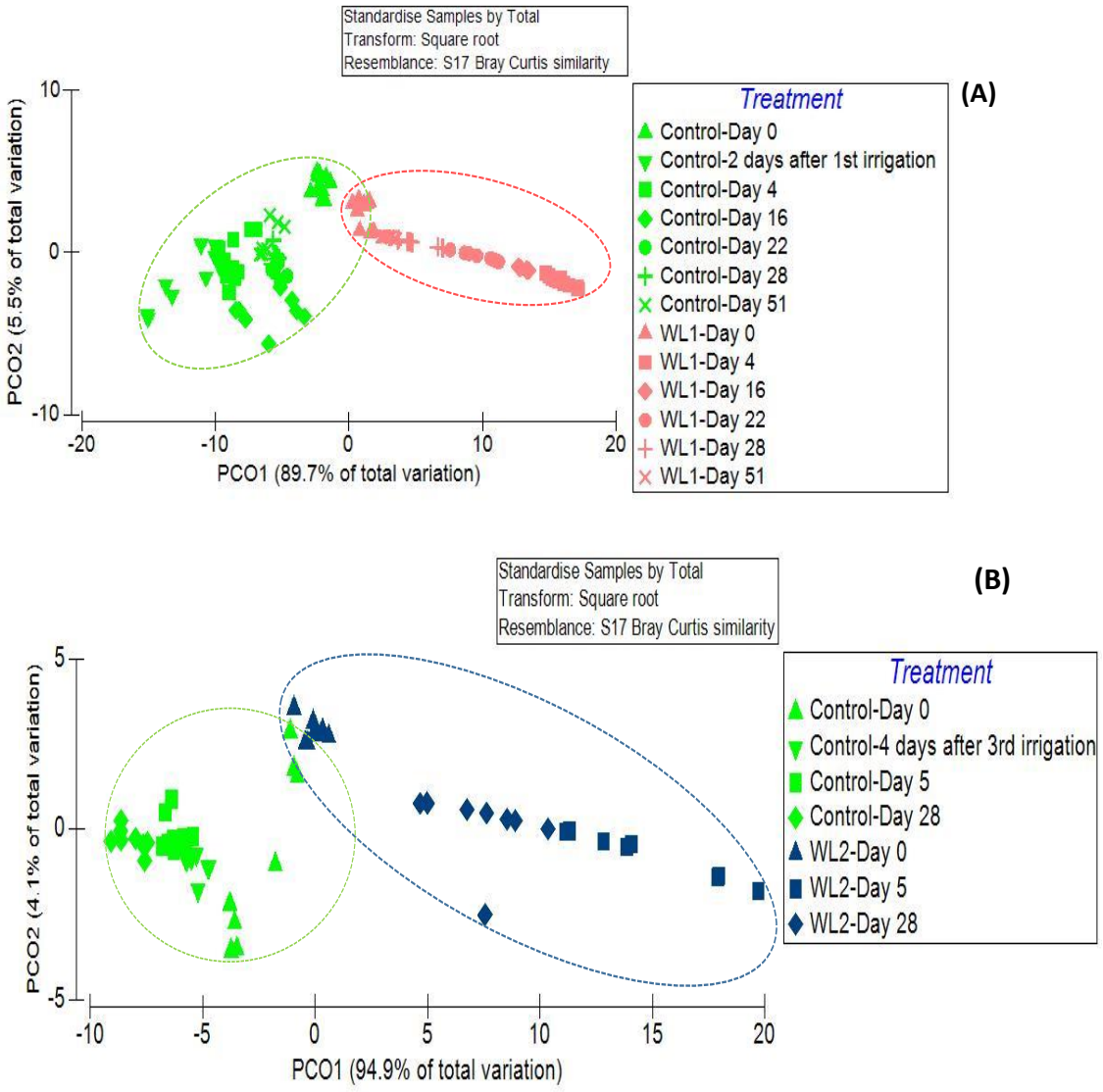

Figure 2.9 Principal coordinates analysis (PCO) derived from the Bray-Curtis dissimilarity matrices showing variations in AOB community structure for (**A**) WL1 and (**B**) WL2 treatments, based on the relative abundance of AOB TRFs. Data are presented for days after treatments. WL1 = Waterlogging 1, WL2 = Waterlogging 2.

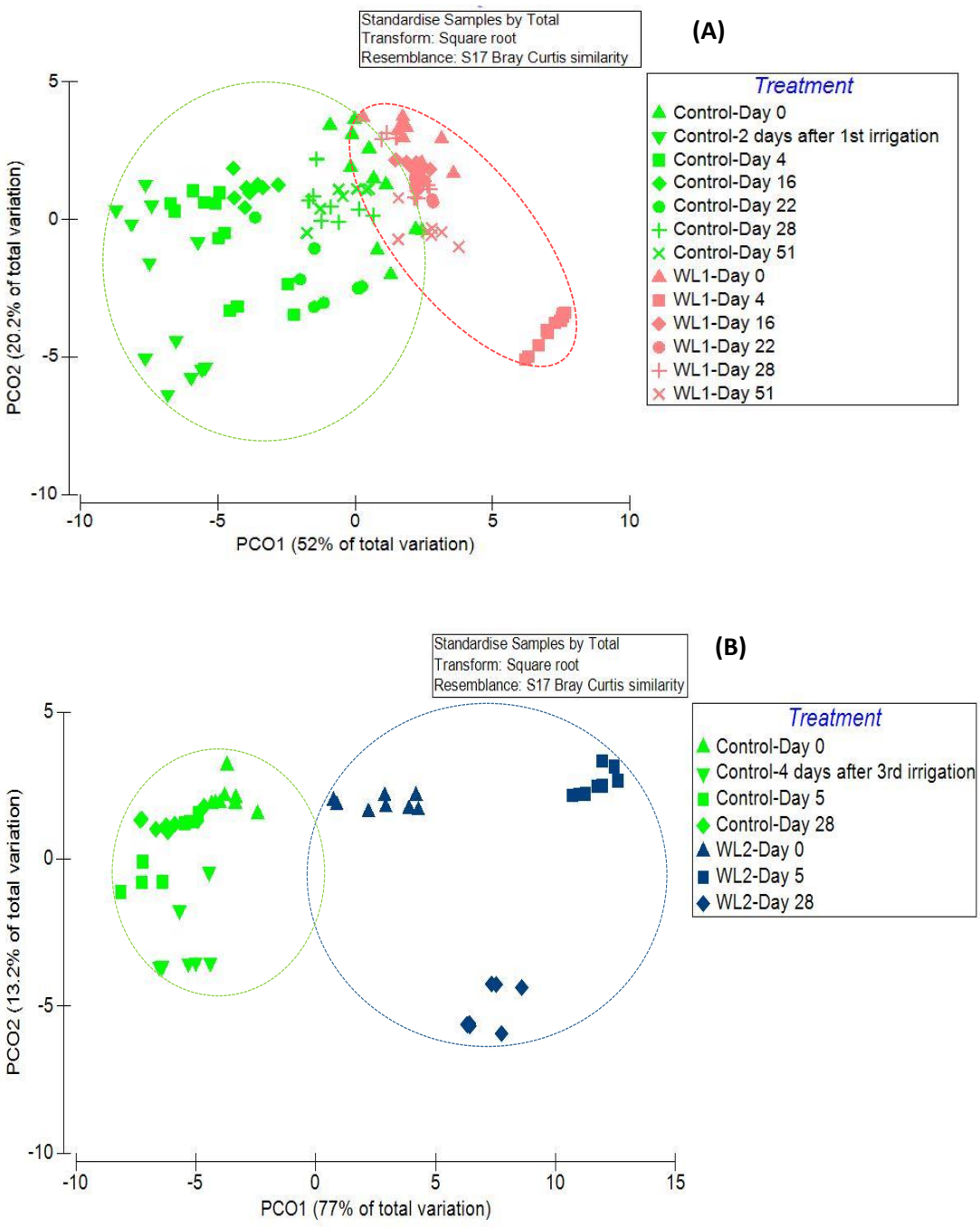

Figure 2.10 Principal coordinates analysis (PCO) derived from the Bray-Curtis dissimilarity matrices showing variations in AOA community structure for (**A**) WL1 and (**B**) WL2 treatments, based on the relative abundance of AOA TRFs. Data are presented for days after treatments. WL1 = Waterlogging 1, WL2 = Waterlogging 2.

Table 2.2 Outputs of PERMANOVA test for treatment effects on AOB community structure, using Type III sums of squares based 999 permutations of residuals under a reduced model. Significant effects are in boldface (P<0.05). WL1 = Waterlogging 1, WL2 = Waterlogging 2. F = The F-value, P = The P-value.

AOB	**Day after treatments**								
	WL1						**WL2**		
	0	**4**	**16**	**22**	**28**	**51**	**0**	**5**	**28**
F	10.653	572.07	591.01	129.71	310.77	297.74	8.5938	200.06	244.96
P	**0.004**	**0.001**	**0.001**	**0.001**	**0.002**	**0.001**	**0.001**	**0.001**	**0.001**

Table 2.3 Outputs of PERMANOVA test for treatment effects on AOA community structure, using Type III sums of squares based 999 permutations of residuals under a reduced model. Significant effects are in boldface (P<0.05). WL1 = Waterlogging 1, WL2 = Waterlogging 2. F = The F-statistic, P = The P-value.

AOA	**Day after treatments**								
	WL1						**WL2**		
	0	**4**	**16**	**22**	**28**	**51**	**0**	**5**	**28**
F	3.6167	126.94	70.702	38.365	43.236	62.284	5.179	127.86	53.085
P	**0.004**	**0.001**	**0.001**	**0.001**	**0.002**	**0.001**	**0.001**	**0.001**	**0.001**

2.3.4 Drivers of process rates

2.3.4.1 Correlation between PNR, AOA and AOB *amoA* genes and soil physicochemical properties

The relationship of PNR, AOB and AOA *amoA* gene abundance, and soil physicochemical properties were examined by Spearman's rank correlation analysis. Overall, there was a strong, statistically significant (r_S=-0.666, P<0.001) linear negative correlation between PNR and soil moisture content (**Table 2.4** & **Figure 2.11**). Waterlogging had effects on the relationship between PNR and soil moisture. In particular, the relationship between PNR and soil moisture of control plots was weaker than that of WL1 and WL2 plots (r_S=-0.582; P<0.001, r_S=-0.696, P<0.001; and r_S=-0.656, P<0.001 for control, WL1 and WL2 plots, respectively, **Table 2.4**).

PNR was also significantly correlated with soil NH_4^+ and pH (r_S=-0.491, P=0.022 and r_S=0.505, P=0.015, respectively). No other significant correlations between PNR and soil NO_3^-, total N were found (**Table 2.4**). There were not much differences in the relationship between PNR and soil NH_4^+ for control and WL1 and WL2 plots (**Table 2.4**).

AOB and AOA *amoA* genes had an overall strong, linear negative correlation with soil moisture (r_S=-0.559, P<0.001 and r_S=-0.517, P<0.001. respectively) (**Table 2.4** & **Figure 2.11**). Stronger relationships between AOA *amoA* genes and soil moisture after waterlogging applied were observed (r_S=-0.439, P<0.001; r_S=-0.620, P<0.001; r_S=-0.763 for control, WL1 and WL2 plots, respectively, **Table 2.4**). Similar trend was found for the relationship between AOB *amoA* gene and soil moisture (r_S=-0.476, P<0.001; r_S=-0.508, P<0.001; r_S=-0.881, P<0.001 for control, WL1 and WL2 plots, respectively, **Table 2.4**).

AOB *amoA* gene abundance was also significantly correlated with soil NH_4^+ and pH (r_S=0.529, P=0.011 and r_S=-0.512, P=0.014, respectively, **Table 2.4**). No significant correlations between AOB abundance and soil NO_3^-, total N; and AOA abundance and pH and soil nutrients (**Table 2.4**). There was little difference in the relationship between AOB *amoA* gene abundance and pH among control, WL1 and WL2 plots (r_S=-0.413, P=0.007;

r_S=-0.404, *P*=0.011; r_S=-0.464, *P*<0.001, respectively). The relationships between AOB *amoA* gene abundance and soil NH_4^+ of WL1 and WL2 plots were slightly weaker than that of control plots (r_S=0.536, *P*<0.001; r_S=0.483, *P*<0.001; r_S=0.461, *P*=0.005 for control, WL1 and WL2 plots, respectively, **Table 2.4**).

Table 2.4 Correlation coefficient of soil physicochemical properties, PNR and the abundance of AOB and AOA for each treatment and all treatments together. Significant differences at *P*<0.01 (**) and *P*<0.05 (*) are in bold. WL1 = Waterlogging 1, WL2 = Waterlogging 2, NH_4^+ = Ammonium, NO_3^- = Nitrate, N = Nitrogen, AOA = Ammonia-oxidizing archaea, AOB = Ammonia-oxidizing bacteria, PNR = Potential nitrification rate.

	Control			**WL1**			**WL2**			**All**		
Variables	**AOA**	**AOB**	**PNR**	**AOA**	**AOB**	**PNR**	**AOA**	**AOB**	**PNR**	**AOA**	**AOB**	**PNR**
Soil moisture	**-0.439***	**-0.476***	**-0.582***	**-0.620****	**-0.508***	**-0.696****	**-0.763****	**-0.881****	**-0.656****	**-0.517***	**-0.559****	**-0.656****
pH	-0.240	**-0.413***	**-0.425***	-0.270	**-0.404***	**-0.478***	-0.250	**-0.464***	**-0.462***	-0.230	**-0.512***	**-0.491***
NH_4^+	-0.246	**0.536***	**0.527***	-0.175	**0.483***	**0.529***	-0.184	**0.461***	**0.513***	-0.245	**0.529***	**0.505***
NO_3^-	-0.177	0.211	0.222	-0.149	0.133	0.231	-0.172	0.136	0.168	-0.201	0.210	0.240
Total N	0.068	0.121	0.161	0.071	0.130	0.143	0.069	0.127	0.157	0.073	0.110	0.135

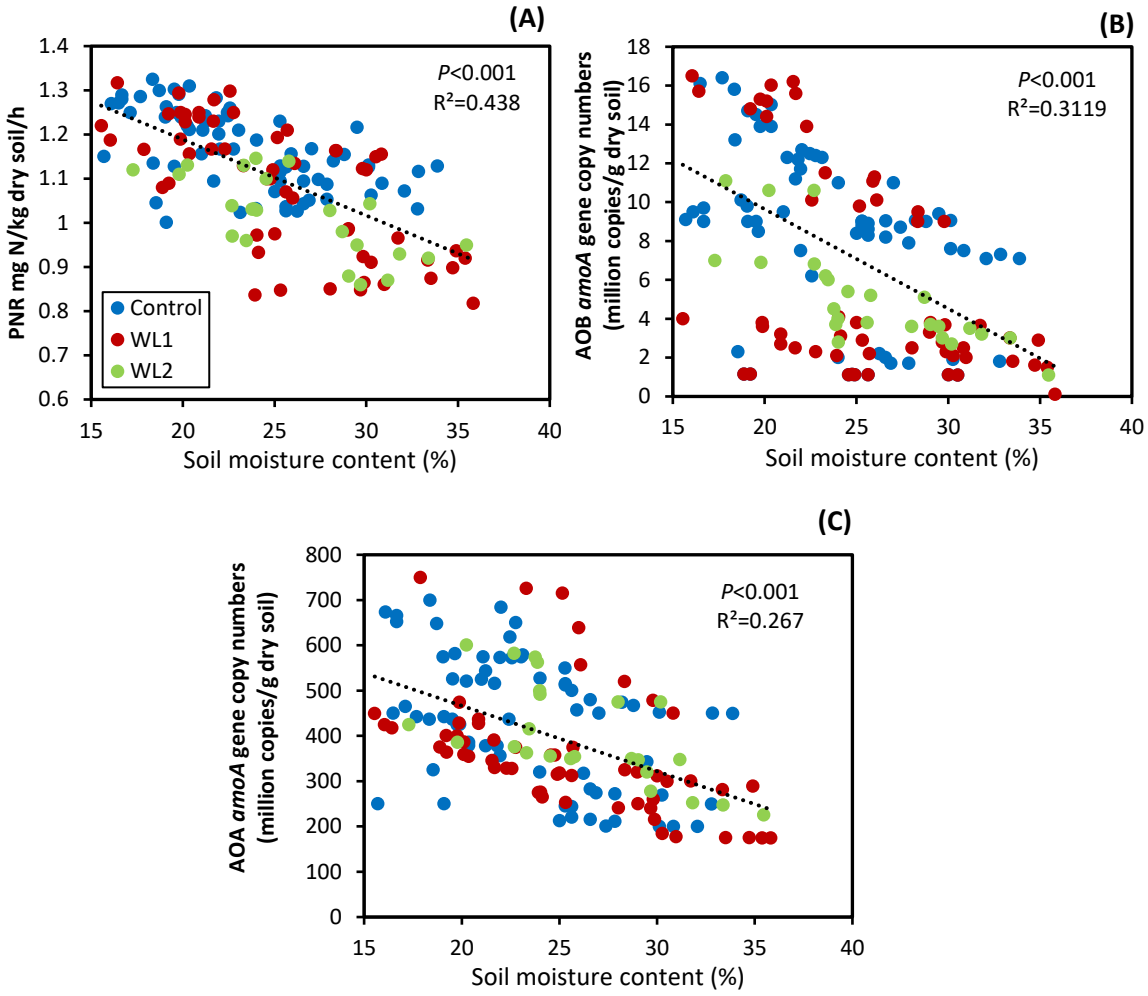

Figure 2.11 Relationship between potential nitrification rate (PNR) (**A**), AOB (**B**), AOA (**C**) *amoA* gene abundance, and soil moisture. WL1 = Waterlogging 1; WL2 = Waterlogging 2.

2.3.4.2 Correlation between potential nitrification rate (PNR) and AOB and AOA *amoA* gene abundance

The relationship of potential nitrification rate (PNR) and the AOB and AOA *amoA* gene abundance were determined by Spearman's rank correlation analysis. Overall, there was

a strong, statistically significant (r_S=0.635, *P*<0.001) linear positive correlation between PNR and AOB *amoA* gene copy number (**Table 2.5 & Figure 2.12**). Similarly, the AOA *amoA* gene abundance was strongly, positively correlated with PNR. Statistical analysis indicated a significant correlation (r_S=0.58, *P*<0.001, **Table 2.5 & Figure 2.12**).

There were also significant linear correlations between PNR and AOB, AOA *amoA* gene abundance for each treatment. The relationship between PNR and AOB *amoA* gene abundance of control plots was not much different from that of WL1 and WL2 plots (r_S=0.668, *P*<0.001; r_S=0.658, *P*<0.001; r_S=0.675, *P*<0.001 for control, WL1 and WL2 plots, respectively, **Table 2.5**). The relationship between AOA *amoA* gene abundance and PNR of control plots was slightly weaker than that of WL1 and WL2 plots (r_S=0.513, *P*<0.001; r_S=0.576, *P*=0.007; r_S=0.589, *P*<0.001 for control, WL1 and WL2 plots, respectively, **Table 2.5**).

Table 2.5 Correlation coefficient of PNR and the abundance of AOB and AOA for each treatment and all treatments together. Significant differences at *P*<0.01 (**) and *P*<0.05 (*).

	Control	**WL1**	**WL2**	**All**
Variables	**PNR**	**PNR**	**PNR**	**PNR**
AOB	0.668**	0.658**	0.675**	0.635**
AOA	0.513*	0.576*	0.589**	0.568*

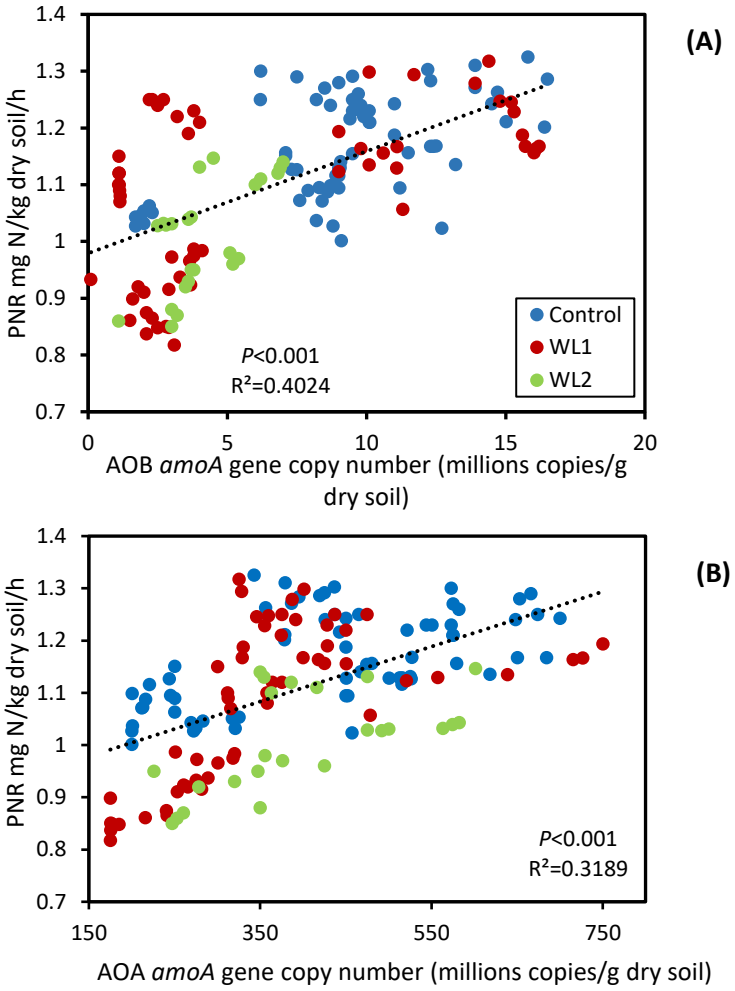

Figure 2.12 Relationship between potential nitrification rate (PNR) and (**A**) AOB, (**B**) AOA *amoA* gene abundance. WL1 = Waterlogging 1; WL2 = Waterlogging 2. AOB = Ammonia-oxidizing bacteria, AOA = Ammonia-oxidizing archaea.

2.3.4.3 Correlation between potential nitrification rate and ammonia-oxidizing community structure

The first five PCOs explained 86.2 % of the variation in the AOB TRFLP pattern; PCO1 and PCO2 accounted for 79.9 % AOB variation. Overall, AOB structure along PCO1 and PCO2 was significantly correlated with PNR (r_S=0.517, *P*<0.001 and r_S=0.375, *P*=0.009, respectively) (**Table 2.6d**). For the AOA community structure, the same trend was observed, but weakly correlated. The first five PCOs explained 95 % of the variation in AOA TRFLP pattern, in which two first PCO axes explained 83.9%. Statistical, significant correlation between AOA structure along PCO1 & PCO2 and PNR was determined with Spearman coefficients r_S=0.261, *P*<0.001, r_S=0.206, *P*=0.012, respectively (**Table 2.7d**).

For each treatment, AOB community structure along PCO1 and PCO2 was also significantly correlated with PNR (r_S=0.624, *P*<0.001 and r_S=0.381, *P*=0.012 for control plots (**Table 2.6a**); r_S=0.615, *P*<0.001 and r_S=0.362, *P*=0.008 for WL1 plots (**Table 2.6b**); and r_S=0.578, *P*<0.001 and r_S=0.368, *P*=0.001 for WL2 plots, **Table 2.6c**). The relationships between AOB community structure and PNR of control plots were slightly stronger than that of WL1 and WL2 plots (**Table 2.6a, b & c**).

In terms of AOA community structure, the same trend was observed. In particular, there were significant correlations between AOA community structure along PCO1 and PCO2 and PNR for each treatment (r_S=0.314, *P*=0.002 and r_S=0.217, *P*=0.008 for control plots (**Table 2.7a**); r_S=0.305, *P*=0.009; r_S=0.211, *P*=0.013 for WL1 plots (**Table 2.7b**); and r_S=0.311, *P*=0.023 and r_S=0.209, *P*=0.017 for WL2 plots, **Table 2.7c**). The relationship between AOA community structure and PNR of control plots was also slightly stronger than that of WL1 and WL2 plots (**Table 2.7 a, b & c**).

Table 2.6 The variation explained, the Spearman rank correlation coefficient (r_S), and the *P* values of correlations between potential nitrification rate (PNR) and AOB community structure for each treatment and all treatments together, as summarized by the top five principal coordinates. Significant effects are in bold (*P*<0.05).

Statistic	PCO1	PCO2	PCO3	PCO4	PCO5
(a) Control					
Variation explained (%)	58.55	29.33	2.17	1.77	1.06
PNR					
r_S	**0.624**	**0.381**	0.127	-0.054	-0.031
P	**<0.001**	**0.012**	0.265	0.781	0.747
(b) WL1					
Variation explained (%)	82.7	3.5	2.55	2.44	1.36
PNR					
r_S	**0.615**	**0.362**	0.103	-0.062	-0.018
P	**<0.001**	**0.008**	0.243	0.764	0.803
(c) WL2					
Variation explained (%)	65.82	12.63	2.02	1.64	1.22
PNR					
r_S	**0.578**	**0.368**	0.095	-0.072	-0.025
P	**<0.001**	**0.007**	0.631	0.711	0.726
(d) all					
Variation explained (%)	66.7	13.2	3.49	1.61	1.24
PNR					
r_S	**0.517**	**0.375**	0.013	-0.075	-0.028
P	**<0.001**	**0.009**	0.877	0.364	0.735

Table 2.7 The variation explained, the Spearman rank correlation coefficient (r_S), and the *P* values of correlations between potential nitrification rate (PNR) and AOA community structure for each treatment and all treatments together, as summarized by the top five principal coordinates. Significant effects are in bold (*P*<0.05).

Statistic	PCO1	PCO2	PCO3	PCO4	PCO5
(a) Control					
Variation explained (%)	58.45	10.02	5.64	3.17	1.38
PNR					
r_S	**0.314**	**0.217**	0.052	-0.211	-0.152
P	**0.002**	**0.008**	0.643	0.781	0.150
(b) WL1					
Variation explained (%)	73.64	8.76	4.23	2.19	1.02
PNR					
r_S	**0.305**	**0.211**	0.063	-0.302	-0.124
P	**0.009**	**0.013**	0.582	0.421	0.761
(c) WL2					
Variation explained (%)	63.85	11.27	5.61	4.23	3.76
PNR					
r_S	**0.311**	**0.209**	0.038	-0.135	-0.107
P	**0.023**	**0.017**	0.654	0.631	0.510
(d) all	70.6	13.28	4.75	3.69	2.71
Variation explained (%)					
PNR					
r_S	**0.261**	**0.206**	0.044	-0.115	-0.148
P	**<0.001**	**0.012**	0.598	0.64	0.073

2.4 Discussion

My findings indicate strong effects of waterlogging on soil physicochemical properties in furrowed irrigated cotton farming, including significant increases in soil moisture and pH, and significant decrease in soil NO_3^- content 4 and 5 days after the first and the second waterlogging events, respectively. The ammonia-oxidizing community was significantly affected by waterlogging. In particular, there were approximately 10-fold decrease in AOB *amoA* gene abundances and shifts in AOB community structure 4 and 5 days after WL1 and WL2, respectively. Similarly, abundance of AOA *amoA* gene decreased by approximately 2-fold and AOA community structure was altered 4 and 5 days after WL1 and WL2, respectively. PNR significantly decreased upon waterlogging. PNR was significantly correlated with both AOB and AOA community abundances and structures; however, the AOB community was more strongly correlated with PNR than the AOA. The significant linear relationship between PNR and ammonia-oxidizing communities suggests that negative effects of waterlogging on these functional microbial communities could result in decreases in nitrification rates, thereby reducing soil NO_3^- availability which is one of important N sources for cotton crop uptake.

2.4.1 Effects of waterlogging on soil physicochemical properties

Waterlogging is known to deplete soil O_2 concentrations by restricting atmospheric gas diffusion into soil (Jackson & Drew, 1984). In this study, significant increases in soil moisture were recorded 4 and 5 days after WL1 and WL2, respectively. It suggested much higher soil moisture during the waterlogging events. Such significant increases in soil moisture during and after WL1 and WL2 events would fill soil gaseous pores, leading to a reduction in soil O_2 concentrations. Decreased soil O_2 levels are expected to negatively affect nitrification (Reddy & Patrick, 1975, Van Schreven & Sieben, 1972) and waterlogging may stimulate leaching, denitrification and anammox (Long *et al.*, 2013), thereby facilitating N loss from agricultural systems. The results presented here support the above observations as both PNR and soil NO_3^- concentrations dropped dramatically in both WL1 and WL2 treatments, suggesting that waterlogging soils had a significant

effect on the soil NO_3^- content at the surface layer (0-10 cm). Such decreases in NO_3^- levels could be a consequence of the combined effect from reduced nitrification plus stimulated NO_3 leaching and denitrification when anoxic conditions were created (Wang & Bettany, 1994, Patrick & Reddy, 1976, Barnard *et al.*, 2005). Such a significant drop of soil NO_3^- caused by waterlogging altered the soil N status, resulting in a potential limitation of plant available N that would be expected to reduce cotton productivity. Previous field study reported a substantial cotton yield loss after exposing soils to waterlogging due to 35% lower radiation use efficiency (Bange *et al.*, 2004). My results provided evidence on soil N reduction caused by waterlogging, thereby potentially contributing to crop nutrient deficiency and decreased productivity. Decreased nutrients, including N, in cotton leaves were also observed after a series of waterlogging events in a study by Milroy *et al.* (2009).

2.4.2. Effects of waterlogging on ammonia-oxidizing communities

During this study, AOA outnumbered AOB across all waterlogging treatments, in agreement with a number of previous studies (Hai *et al.*, 2009, Sterngren *et al.*, 2015). Ammonia-oxidizer abundance and structure changed significantly after waterlogging. These alterations are likely due to changes in soil moisture, which is a key factor affecting nitrifying microorganisms. It has been demonstrated that waterlogging quickly depletes soil O_2 concentrations by restricting atmospheric gas diffusion into soils, resulting in hypoxic/anoxic environments (Jackson & Drew, 1984) that are unfavorable for ammonia-oxidizing communities. I observed considerable increases in soil moisture after waterlogging treatments were applied. This provided evidence for an increase in water-filled pores in the soils, leading to a reduction in soil O_2 concentrations. Such decreases in soil O_2 levels would negatively affect nitrifying microorganisms possessing ammonia-oxygenase enzymes which are highly dependent on O_2 availability (Belser, 1979). This is comparable with my findings where the abundance of AOB and AOA *amoA* genes decreased upon waterlogging. Additionally, TRFLP analysis indicated AOB TRF-55 and AOA TRF-74 significantly decreased after waterlogging treatments were applied. Strong

correlations between the abundance of AOB and AOA *amoA* genes and soil moisture content were also observed in this study.

Soil pH has been indicated to be a major factor affecting ammonia-oxidizing communities and their activity (Gubry-Rangin *et al.*, 2011, Nicol *et al.*, 2008). The decreased AOB and AOA abundances and alterations in their community structures observed in this study may be the consequence of changes in soil pH. In this study, soil pH increased up to 8.2 following waterlogging, which could be the consequence of CO_2 accumulation in the soil since waterlogging fills soil pores with water so that the passage of CO_2 through the soil is blocked, resulting in the formation of bicarbonate ions (Greenway *et al.*, 2006). Lu *et al.* (2004) also found that soil pH increased as a consequence of waterlogging and such elevation was regardless of initial soil pH. Interestingly, pH of control soils dropped to 7.51 at the end of the experiment while that of WL1 and WL2 soils were 7.95 and 7.84, respectively. However, no significant differences in soil pH between control, WL1 and WL2 plots at Day 28 and 5 after WL1 and WL2, respectively were observed. Since rainfall occurred at Day 39 (28 mm) and 41 (48 mm) after WL1 which were in turn corresponding to Day 16 and 18 after WL2, leaching of soil base cations might be facilitated. Such an increase in base cation leaching could lower soil pH (McCauley *et al.*, 2009). No proper answer for the decreased pH of control soils was given in my study; however, one potential explanation may be due to a higher amount of cation leaching in control plots when compared to WL1 and WL2 plots. AOB driven nitrification has been known to favor pH ranges from neutral to slightly alkaline (Gieseke *et al.*, 2006), thus pH values higher than 8.0 after WL1 and WL2 might result in a change in abundances of AOB. AOA have been demonstrated to be favored in various acidic soils (Yao *et al.*, 2011, Zhang *et al.*, 2012a), and hence strongly alkaline pH values upon waterlogging might negatively affect the AOA community.

Different responses of AOB and AOA abundances to waterlogging treatments were found in this study. In particular, the copy number of AOB *amoA* genes dropped about 10-fold whereas that of AOA *amoA* genes decreased less. Differential responses of AOA

and AOB to waterlogging could be due to the dissimilarities of their biochemical and genetic characteristics (Offre *et al.*, 2014, Webster *et al.*, 2005). AOB are Gram-negative bacteria having thin peptidoglycan cell walls which make them more sensitive than AOA to disturbances such as changes in soil moisture (Schimel *et al.*, 2007). AOA has less permeable membranes making them more stable when exposed to water stresses (Valentine, 2007). This study provides novel evidence that both AOA and AOB communities were responsive to waterlogging, but AOB are more susceptible to water stress than AOA in furrowed irrigated cotton soils. Several previous studies have reported responses of ammonia-oxidizers to increased soil moistures; however, only AOB or AOA were responsive to changes in soil water availability (Gleeson *et al.*, 2010, Szukics *et al.*, 2012, Chen *et al.*, 2013). For example, only AOA responded to a 75% increase in water-filled pore space (WFPS) in a forest soil (Szukics *et al.*, 2012), whereas, the study by Chen *et al*., (2013) observed only AOB responses to increased precipitation in a temperate steppe. Therefore, my findings further support the differences in physiological features and ecological niches of AOA and AOB.

The phylogenetic affiliation to some AOB and AOA TRFs could be assigned, based on the study by Hu *et al.* (2015), in which AOA TRF-74 belonging to the *Nitrososphaera* cluster was affected by waterlogging, suggesting that *Nitrososphaera* could be vulnerable to water stress. Previous work has indicated that *Nitrososphaera* is the most abundant AOA cluster and is functionally active in autotrophic nitrification in acidic and alkaline agricultural soils (Hu *et al.*, 2013, Xia *et al.*, 2011, Zhang *et al.*, 2012a). My findings are in agreement with these studies as TRF-74 was one of the most abundant TRFs. In terms of the AOB community, TRF-55 was also significantly reduced upon waterlogging; however, its phylogenetic affiliation could not be identified.

2.4.3 Relationship between potential nitrification rate and ammonia-oxidizers

Environmental change can impact soil process rates by multiple mechanisms (Singh *et al.*, 2010). For example, it can change soil physicochemical properties which can directly impact the microbial process rate. Alternatively, environmental change can also directly

impact the metabolic rate of the microbial communities or indirectly control the process rate by impacting the community structure and relative abundance (Schimel *et al.*, 2007). Identifying which microbial mechanism explains the shift in process rate in response to environmental change is considered a real challenge in microbial ecology. However, such knowledge is critical to incorporate microbial properties (e.g. abundance, diversity) in simulation models to improve predictions and in management policy to minimize the impact on ecosystem services including crop productivity (Singh *et al.*, 2010). In this study, there were strong correlations between potential nitrification rate (PNR) and the abundance and structure of ammonia-oxidizing communities, suggesting that both abundance and community structure were important determinants of PNR and the response of PNR to waterlogging. It also suggests that nitrification is driven by both AOA and AOB in furrow-irrigated cotton soil. These results are supported by previous studies which found strong relationships between the ammonia-oxidizers and their function. In particular, AOA were found to drive nitrification in acidic soils in the study by Yao *et al.* (2011), whereas Di *et al.* (2009) found that AOB were drivers of nitrification in nitrogen-rich grassland soils. In semi-arid agricultural soil, AOB was found to be dominant in nitrification activity (Banning *et al.*, 2015). Taken together, it suggests that in agricultural fields where N fertilizers are added, AOB seems to be the major driver of nitrification rates. However, in harsh environmental conditions, such as acidic soils with low substrate availability, AOA outcompete AOB in terms of activity (Yao *et al.*, 2011, Di *et al.*, 2009, Hu *et al.*, 2015). In this study, no clear niche differentiation was observed. Additionally, negative linear relationships between PNR, the abundance of AOA and AOB, and soil moisture in this study (**Figure 2.10**) supports my hypothesis that waterlogging changed soil physicochemical properties, resulting in shifts in ammonia-oxidizing communities and negative impacts on nitrification rate.

2.5 Conclusions

By employing a polyphasic approach to combine soil physicochemical properties, PNR and molecular methodology, this study provides evidence that waterlogging created

unfavorable conditions such as increased soil water content leading to hypoxic or even anoxic environments that would affect the nitrification process. I found a decline of AOB and AOA abundances, in addition to alterations of community composition upon waterlogging treatments. Changes in potential nitrification rates were correlated with ammonia-oxidizing communities, suggesting a strong link between ammonia-oxidizers and their functioning in the furrow-irrigated cotton soil. I also found the AOB response was greater than AOA to waterlogging, supporting the theory of different physiology and ecological niches between AOB and AOA. In terms of the relative contribution of AOB and AOA to nitrification, I did not observe functional redundancy since both AOB and AOA were strongly correlated with specific functions.

Regarding soil nutrients in response to waterlogging, my results showed a significant reduction in soil NO_3^- which is required for plant growth and development. These decreases could be a consequence of reduced nitrification, in combination with stimulated NO_3^- leaching and denitrification, when anoxic conditions were created due to waterlogging. However, I did not examine NO_3^- leaching and denitrification in response to waterlogging in this study. Soil N depletion resulting from waterlogging is expected to negatively affect cotton crop productivity.

Overall, my findings provided novel insights into the impacts of waterlogging on the relationship between soil microbial communities and their functions. These results may allow us to better predict the response of agro-ecosystems such as cotton farming to global change including the projected higher frequency and intensity of extreme weather events, thereby developing effective N management strategies to maintain high crop productivity.

Waterlogging depletes soil N availability which is related to crop productivity, and hence external N supply may improve soil fertility and help cotton plants recover (Goswami, 1990, Wen-qi *et al.*, 2010). It is crucial to determine application method, rate, and timing to alleviate negative impacts of waterlogging on soils and plants. Previous studies have recommended that the application of fertilizers 5 or 8 days after waterlogging

significantly increased growth and yield of cotton (Li *et al.*, 2013, Wu *et al.*, 2012). Additionally, foliar N application has been suggested to diminish deleterious effects of waterlogging on cotton lint yields (Hodgson & MacLeod, 1988). Overall, the external N supply will be an effective approach to help soils and plants recover upon exposure to waterlogging stress.

CHAPTER 3 IMPACTS OF ELEVATED CO_2 AND TEMPERATURE ON SOIL NITRIFICATION AND AMMONIA-OXIDIZING COMMUNITIES IN COTTON FARMING

3.1 Introduction

Anthropogenic activities have continued to increase greenhouse gas emissions into the atmosphere. The current atmospheric CO_2 concentration of 400 ppm is approximately 1.5-fold higher than the 250-280 ppm concentrations experienced during the pre-industrial period (Monastersky, 2013). Simultaneously, the global surface temperature has experienced an increase of 0.74^o C during the twentieth century, and is projected to rise another 1.1^o to 6.4^o C by the end of 21st century (IPCC, 2013). These global climate changes are expected to have considerable effects on agricultural ecosystems and their functions such as biomass productivity and nutrient cycling (Rosenzweig *et al.*, 2007). As an example, climate change may affect soil moisture levels (Chiew *et al.*, 1995, Kirkham, 2016), thereby altering C and N availability in soils (Gleeson *et al.*, 2010, Barnard *et al.*, 2013), which in turn can influence crop nutrient acquisition and yields. Global crop productivity has been projected to increase by approximately 1.8% in response to elevated CO_2 and decrease roughly 1.5% in response to warming over the next few decades (Lobell & Gourdji, 2012). However, climate change will include simultaneous increases in CO_2 and temperature, and projected changes in crop productivity due to the interactive effects of these two variables are known.

Elevated CO_2 has been shown to enhance net primary productivity through stimulating plant photosynthesis and root production which results in an increase to labile C levels in the soil, and lower stomatal conductance leading to increased plant water use efficiency (Lawlor & Mitchell, 1991, Bhattacharyya *et al.*, 2013, Kimball *et al.*, 2002).

Warming can increase or decrease net plant productivity, depending on the optimal temperature range (Trumbore, 1997, Ganpat, 2014).

Australian cotton crops may be vulnerable to climate change (Glover *et al.*, 2008), especially to rising temperature since cotton is grown in high temperature regions and strongly depends on these temperatures to maintain high productivity (Reddy *et al.*, 1997). Although no clear common temperature range for optimal cotton growth has been determined, the thermal kinetic window in which upland cotton often exhibits the most efficient metabolic activities is between 23.5 to 32^{o}C (Burke *et al.*, 1988). For example, the optimal growth for cotton leaves and stems is approximately 30^{o}C (Hodges *et al.*, 1993); when temperatures exceeded 35^{o}C, leaf area has been shown to decrease significantly (Reddy *et al.*, 1992). Temperatures exceeding 35^{o}C lead to a significant decrease in cotton photosynthesis, as observed in the study by Bibi *et al.* (2008).

In terms of elevated CO_2, cotton plants often respond positively by increasing photosynthesis, water use efficiency and biomass, potentially resulting in enhanced crop productivity. For example, photosynthetic rate increased by 30-34% when cotton was exposed to CO_2 concentrations between 500-900 ppm (Idso *et al.*, 1994). In addition, cotton plant biomass and yield increased by approximately 37% and 43%, respectively when grown at 550 ppm CO_2 (Mauney *et al.*, 1994). However, high temperatures may negate the CO_2 fertilization benefits to cotton crops (Reddy *et al.*, 2005). As a consequence, current models based on a single climate factor to predict potential effects of future climate on crop yields, may be limited as the interactive effect of increasing CO_2 and temperature may be very complex (Sun *et al.*, 2012, Yoon *et al.*, 2009).

Soil nutrient cycling is mainly driven by microbial communities, which provide key nutrients for plant growth (Singh *et al.*, 2010). The nitrification process is controlled by AOB and AOA and plays an important role in contributing to plant N availability (both natural and inorganic N fertilizer-added ecosystems), N loss through gas emission into the atmosphere and NO_3^- leaching to groundwater (Zhalnina *et al.*, 2012), thereby indirectly influencing crop productivity. Increasing atmospheric CO_2 concentrations are

expected to indirectly affect nitrification rates via plant-mediated impacts on soil properties (Singh *et al.*, 2010). Soil substrate availability may be changed under elevated CO_2 due to alterations in NH_4^+ production rates caused by an increase in root exudates and plant residues and/or competition for NH_4^+ between nitrifiers and other NH_4^+ consumers, particularly where N is limited such as in non-fertilized dryland ecosystems (Niboyet *et al.*, 2010, Hu *et al.*, 2015). In addition, elevated CO_2 may alter soil O_2 concentrations, an important factor controlling nitrification efficiency, by reducing plant stomatal conductance, and hence increasing soil water content, thereby altering rates of nitrification (Barnard *et al.*, 2006).

Temperature has been demonstrated to be a key factor affecting nitrification efficiency and for shaping AOB and AOA distribution patterns in the soils (Tourna *et al.*, 2008, Cao *et al.*, 2013, Fierer *et al.*, 2009). Elevated temperatures can affect nitrification through changes in soil and plant properties, metabolic activities, or community structure and abundance of nitrifiers (Hu *et al.*, 2016). In particular, elevated temperature often increases N mineralization and may induce plant biomass, thereby potentially altering soil substrate availability which controls nitrification rates (Bai *et al.*, 2013, Hu *et al.*, 2016). Soil microbial respiration may be increased in response to elevated temperature, resulting in alterations in soil O_2 status which may affect the aerobic ammonia oxidation step (Bradford *et al.*, 2008). Given that microbially-mediated nitrification depends on temperature-sensitive enzyme activities, the process rate can be directly affected by warming through temperature-induced metabolic activities of microorganisms (Karhu *et al.*, 2014). On the other hand, elevated temperatures may also alter AOB and AOA community structure and abundance, resulting in the modification of fundamental physiologies driving process rates. However, it remains largely unclear whether and how nitrifying community structure and abundance will be impacted by climate factors.

Previous work has primarily focused on addressing the response of soil N dynamics and above-ground plant properties to climate change in ecosystems such as grasslands, forests, and crop lands (Rütting *et al.*, 2010, Phillips *et al.*, 2011, Ghannoum *et al.*, 2010,

Dijkstra *et al.*, 2012, Fujimura *et al.*, 2012), whereas less effort was made to fully understand below-ground microbial population dynamics, particularly ammonia-oxidizing communities, in response to increasing CO_2 and temperature. In a growth-chamber study by Hu *et al.* (2016), effects of elevated CO_2 and temperature on nitrification rates and nitrifying communities were elucidated using soils collected from natural dryland sites with *Eucalyptus* trees as the main vegetation. In this study, climate warming exhibited significant direct and indirect effects on the nitrification rate through stimulated AOA metabolic activity, substrate availability and shifts in AOA community abundance and structure. However, no obvious effects of elevated CO_2 were observed, potentially due to the short duration of the experiment. Additionally, a strong effect of elevated temperature on nitrification was shown to be slightly alleviated by elevated CO_2 in dryland soils when they were combined in a study by Hu *et al.* (2016). The evidence reflected niche differentiation of AOA and AOB in environments with low availability of nutrients in response to climate change.

In agroecosystems, soils are well-watered and N fertilized, suggesting that ammonia-oxidizers may respond differently. To date, a limited number of studies on the responses of soil nitrification to climate change in agricultural soils have reported inconsistent data. For example, no significant effect of elevated CO_2 on the nitrification rate or AOB and AOA communities were observed in a soybean free air enrich experiment (FACE) (Pereira *et al.*, 2011, Pereira *et al.*, 2013). In contrast, elevated CO_2 was reported to significantly increase AOB and AOA abundance, and the rate of nitrification in a rice field study (Liu *et al.*, 2015). The study by Liu *et al.* (2015) also found warming did not affect soil nitrification rates and ammonia-oxidizers in a rice paddy field, whereas negative effects of elevated temperature on nitrification rates were observed in a pot trial containing wheat (Rakshit *et al.*, 2012). Such varied responses of soil nitrification and nitrifiers to climate change suggest the importance of factors such as soil type and crop species.

Given that cotton productivity is strongly dependent on large amounts of N-based fertilizers and is vulnerable to climate change, our understanding of nitrification and the

ammonia-oxidizer response to climate change in agricultural soils, particularly in cotton farming systems, is largely unknown. Therefore, this study was conducted using field-based, environmentally-controlled chambers to simulate elevated CO_2 and temperature at the Australian Cotton Research Institute (ACRI) in Narrabri to evaluate the response of soil nitrification and ammonia-oxidizing communities to climate change in cotton crop systems. The outcomes of this study will provide insights into the effects of projected climate change on soil N availability which is directly related to cotton crop productivity, thereby potentially contributing to the development of effective N management strategies to sustain high crop yields. To achieve these objectives, I hypothesized that (i) elevated temperature will increase the nitrification rate through the stimulation of metabolic activities of ammonia-oxidizers, or through changes in soil properties, ammonia-oxidizing community structure and abundance; and (ii) elevated CO_2 will indirectly affect the nitrification rate through plant-mediated changes in soil properties and ammonia-oxidizer community structure and abundance.

3.2 Materials and methods

3.2.1 Field site

This field experiment was conducted at the Australian Cotton Research Institute (ACRI) at Narrabri (30.31^{o}S, 149.78^{o}E) in north-west New South Wales, Australia. Details of climatic and soil characteristics of this region were described in Chapter 2 (section 2.2.1).

In this experiment, cotton was sown in late summer (February, 2015) and the field experiment was finalised by the middle of autumn (April, 2015). Mean maximum and minimum daily temperatures during this period were 30^{o}C and 16^{o}C, respectively (Bureau of Meteorology, NSW).

3.2.2 Cotton cultivation

The cotton cultivar used in this study was CSIRO Sicot 71BRF. The N fertilizer application was described in Chapter 2 (section 2.2.2). Irrigation during cotton growth is described below in section 3.2.3.

3.2.3 Experimental design

The field-based environmentally-controlled chambers were constructed of galvanized steel attached to a 1 cm thick transparent plastic segment at the front and back. The chambers were enclosed with two layers of transparent plastic sheets (100 µm thick), which were inflated during the experiment. The chamber size was 4 x 4 x 3 meter (**Figure 3.1A**). The chambers were portable and installed in the field when cotton plants were at the emergence stage (23 days after planting). Two chambers were set for ambient atmospheric CO_2 and elevated temperature (C_aT_e), and two chambers were set for elevated CO_2 and elevated temperature (C_eT_e). Two plots outside the chambers (C_aT_a) were used as field controls (**Figure 3.1B**).

An air conditioner unit was used to maintain air temperature inside the chambers between 2-4^oC higher than ambient air temperature. CO_2 gas was released into the chambers to maintain the CO_2 concentration at ambient (400 ppm) and elevated (550 ppm) CO_2. The concentration of CO_2 inside each chamber was recorded using a LICOR-840A. The temperature and humidity inside the chambers were measured by a Tiny Tag Ultra sensor (Gemini Data Loggers, West Sussex, UK) placed just above the canopy.

Plots were furrow irrigated three times during the experiment. The first irrigation event was applied at 1 day after planting (DAP), the second event at 23 DAP, and the third event at 50 DAP. Each furrow irrigation event provided approximately 90-100 mm water. Drip irrigation systems were set up inside the chambers to ensure similar watering regimes in both the outside plots and inside the chambers; however, drip irrigation was also applied twice for both plots inside and outside chambers at 34 and 35 DAP, providing 21.2 mm water for plots inside chambers and 19.6 mm water for control plots.

Additionally, drip irrigation was applied only for plots inside chambers at four different times: 37 DAP (5.4 mm water), 49 DAP (13.2 mm), 55 DAP (12 mm) and 62 DAP (11 mm), in order to match outside rainfall events.

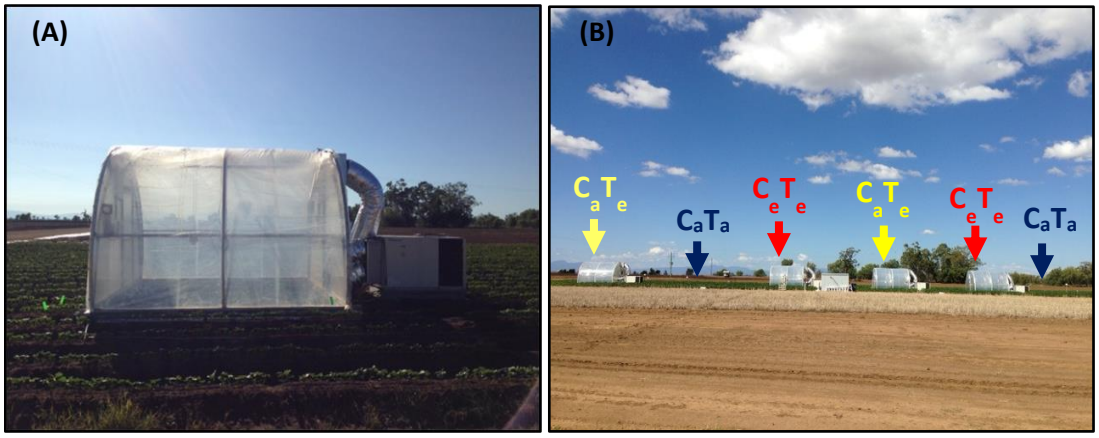

Figure 3.1 Photo of a field-based chamber **(A)** and the field experiment layout **(B**) at ACRI in Narrabri, NSW. C_aT_a = Ambient CO_2 and temperature, C_aT_e = Ambient CO_2 and elevated temperature, C_eT_e = Elevated CO_2 and elevated temperature.

3.2.4 Soil sampling

Soil core samples (4 cm diameter and 10 cm deep) were collected four times during the growing season, representing different developmental stages of the cotton plants. The first sampling campaign was conducted before the chamber installation, corresponding to 15 DAP, when cotton plants were at the emergence stage. The other three sample collection campaigns were conducted 4 days after chamber installation (27 DAP), at the early squaring stage (41 DAP) and the early flowering stage (70 DAP). Five samples were collected each time for plots inside and outside the chamber. Soil samples were transferred to the Hawkesbury Institute for the Environment (HIE), Western Sydney University, NSW for analyses. At HIE, soil samples were passed through a 4 mm sieve to

remove plant residue, then kept at 4^{o}C until analysis. Subsamples of soil were kept at -20^{o}C prior to molecular manipulations.

3.2.5 Soil physicochemical properties analyse

Soil moisture content, pH, NH_4^+, NO_3^- and total C and N were determined according to the detailed procedures described in Chapter 2 (section 2.2.5).

3.2.6 Potential nitrification rate

Potential nitrification rate was measured using the chlorate inhibition method from Kandeler & Böhm (1996). The detailed protocol was described in Chapter 2 (section 2.2.6).

3.2.7 Microbial community analysis

3.2.7.1 DNA extraction

Total soil genomic DNA was extracted using the MoBio PowerSoil DNA Isolation kit (MoBio Laboratories, Carlsbad, CA, USA). The procedures were described in detail in Chapter 2 (section 2.2.7.1).

3.2.7.2 Quantitative PCR

The abundances of bacterial and archaeal *amoA* gene (AOB and AOA) were quantified by real-time PCR. The details of methods including specific primer pairs, PCR reaction components and thermal conditions were described in Chapter 2 (section 2.2.7.2). In this experiment, PCR efficiency for different assays ranged between 86% and 97%.

3.2.7.3 Terminal restriction fragment length polymorphism

The community structure of AOB and AOA were analyzed by using TRFLP. AOB and AOA *amoA* gene fragments were amplified using fluorescently labelled primers FAM-CrenamoA23f/CrenamoA616r and VIC-amoA-1F/amoA-2R respectively. PCR reaction

components and thermal conditions were described in detail in Chapter 2 (section 2.2.7.3).

PCR product purification and measurement of DNA concentration were conducted according to the method presented in Chapter 2 (section 2.2.7.3). The concentration of purified DNA ranged from 40 to 100 ng/µl. The ratio of $A_{260/280}$ and $A_{260/230}$ varied in the range of 1.8-2.1 and 0.7-1.7, respectively.

Purified PCR products were then subjected to digestion by commercial restriction enzymes including MspI and HpyCH4V (NewEngland BioLabs, USA) for AOB and AOA, respectively. Details of digestion protocol and terminal restriction fragment analyses have been previously mentioned in Chapter 2 (section 2.2.7.3).

3.2.8 Statistical analysis

Repeated measures ANOVA was applied to test whether there were significant treatment and plant developmental stage effects on soil properties, PNR, AOB and AOA *amoA* genes. One-way ANOVA with Tukey's HSD test were applied to compare means of different climate change treatments at each plant developmental stage. Spearman's rank correlation analysis was conducted to examine the relationship between the abundance and structure of AOB and AOA and PNR; soil physicochemical properties, PNR and ammonia-oxidizing communities. The *amoA* gene copy numbers were log-transformed prior to statistical analysis to meet normality assumptions. $P<0.05$ was considered to be statistically significant. All these tests were manipulated in SPSS 22 (IBM, Armonk, NY, USA).

The Bray-Curtis dissimilarity matrices based on the relative abundance of AOA and AOB TRFs was visualized by principal coordinate analysis (PCO) using Primer v6 (PRIMER-E Ltd, Plymouth, UK), following PERMANOVA to examine the significance of Bray-Curtis dissimilarity.

3.3 Results

3.3.1 Chamber effects and treatments

The average daily air temperature of C_aT_a plots was 24.2°C while that of C_aT_e and C_eT_e were 25.3°C and 25.1°C, respectively (**Figure 3.2A**). No statistically significant differences in average daily air temperature were observed between C_aT_e and C_eT_e treatments (*P*=0.35). In contrast, the average daily temperature of C_aT_a plots was significantly lower than C_aT_e (*P*=0.012). The daily average vapor pressure deficit (VPD) between C_aT_a and C_aT_e was significantly different (*P*=0.011, **Figure 3.2B**). Similarly, there was significant difference in VPD between C_aT_e and C_eT_e (*P*=0.009, **Figure 3.2B**). Photosynthetically active radiation (PAR) inside and outside chamber was measured only for C_aT_e treatment. Significant differences in PAR between inside and outside chamber were observed (*P*=0.008, **Figure 3.2C**).

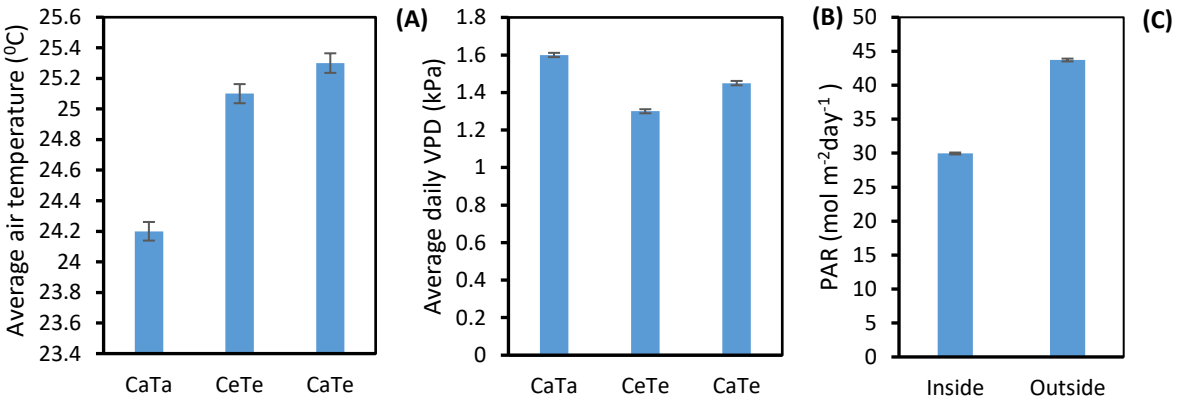

Figure 3.2 Average daily temperature (**A**) and VPD (**B**) among climate treatments during the experiment from 9/2/2015 to 16/4/2015. The average daily PAR between inside and outside C_aT_e chamber (**C**) during the experiment from 11/3/2015 to 13/4/2015. Values represent mean ± SE (n=67 for air temperature and average daily VPD; n=34 for PAR) of each treatment. C_aT_a = Ambient CO_2 and temperature, C_eT_e = Elevated CO_2 and temperature, C_aT_e = Ambient CO_2 and elevated temperature. VPD = Daily average vapor pressure deficit, PAR = Photosynthetically active radiation.

3.3.2 Soil physicochemical properties

Soil moisture varied in the range of 16.5-23.1% across all treatments and crop developmental stages (**Figure 3.3A**). One-way ANOVA and Tukey's HSD indicated that there were significant treatment effects of C_aT_e on soil moisture at seedling establishment and early flowering (*P*=0.01 and *P*=0.027, respectively). Similarly, there was a significant difference in soil moisture between C_aT_e and C_eT_e chamber plots at the early flowering stage (70DAP) (**Figure 3.3A**). Repeated measure ANOVA indicated significant effects of crop developmental stage and C_eT_e on soil moisture (*P*<0.001 and *P*=0.001, respectively). In contrast, no significant effect was observed for C_eT_e, the interaction of C_aT_e and time, and C_eT_e and time (**Table 3.1**).

Soil pH ranged from 7.2 to 7.8 across all treatments and developmental stages (**Figure 3.3B**). An upward trend of soil pH was observed during the growing season. Soil pH increased to approximately 7.8 after 70 DAP corresponding to the early flowering stage. One-way ANOVA and followed Tukey's HSD test showed no significant effect of treatments on soil pH at each developmental stage. Repeated measure ANOVA showed that crop growth stages significantly affect soil pH (*P*=0.002) (**Table 3.1**). C_aT_e and the interaction of C_aT_e and time had no significant effect on soil pH (*P*=0.31 and *P*=0.08, respectively). C_eT_e and its interaction with time also did not significantly affect soil pH (*P*=0.262 and *P*=0.09, respectively) (**Table 3.1**).

Soil inorganic N decreased during the growing season. In particular, soil NH_4^+ concentration dropped from 12 mg/kg dry soil to approximately 6 mg/kg dry soil at the early flowering stage (70 DAP). No significant treatment effect was observed at any development stage (**Figure 3.3C**). Repeated measures ANOVA indicated a significant effect of crop developmental stages on soil NH_4^+ concentration (*P*=0.004). However, no significant effect was observed for C_aT_e, C_eT_e and their interactions with crop developmental stages (**Table 3.1**).

A decrease in soil NO_3^- was also observed for the duration of the experiment across all treatments (**Figure 3.3D**). Soil NO_3^- of ambient and C_aT_e plots were decreased 37% and 44%, respectively whereas that of C_eT_e plots were reduced 53% after 70 DAP corresponding to the early flowering stage. One-way ANOVA showed that there was no significant difference in soil NO_3^- between C_aT_a and C_aT_e treatments (P>0.05) at all developmental stages. In contrast, soil NO_3^- levels at C_eT_e were significantly different than C_aT_e (P<0.001) after 70 DAP corresponding to the early flowering stage whereas no significant difference was observed among all treatments at the other developmental stages (**Figure 3.3D**). Repeated measures ANOVA showed that crop developmental stage, C_eT_e and its interaction with crop developmental stage, on soil NO_3^- concentration were statistically significant (P<0.001 and P=0.035). In contrast, no significant effect of warming and its interaction with time was observed (**Table 3.1**).

Soil total N decreased during the time course of the experiment (**Figure 3.3E**). The amount of soil total N decreased approximately 10-13 % after 70 DAP. One-way ANOVA indicated no significant difference of soil total N among all treatments at each developmental stage (**Figure 3.3E**). Only crop growth had significant effect of soil total N (P=0.001), whereas no significant effect of C_aT_e, C_eT_e and their interaction with crop growth was observed (**Table 3.1**).

Soil total C also changed during the experiment. Soil total C varied from 11.59 g/kg dry soil to 11.91 g/kg dry soil across all treatments and crop growth stages (**Figure 3.3F**). Under C_aT_a, C_aT_e and C_eT_e treatments, soil total C decreased approximately 2.25%, 1.69% and 1.08%, respectively after 70 DAP. Repeated measure ANOVA indicated no significant effects of time, and elevated CO_2 x time on total C (P=0.134 and P=0.646, respectively) (**Table 3.1**).

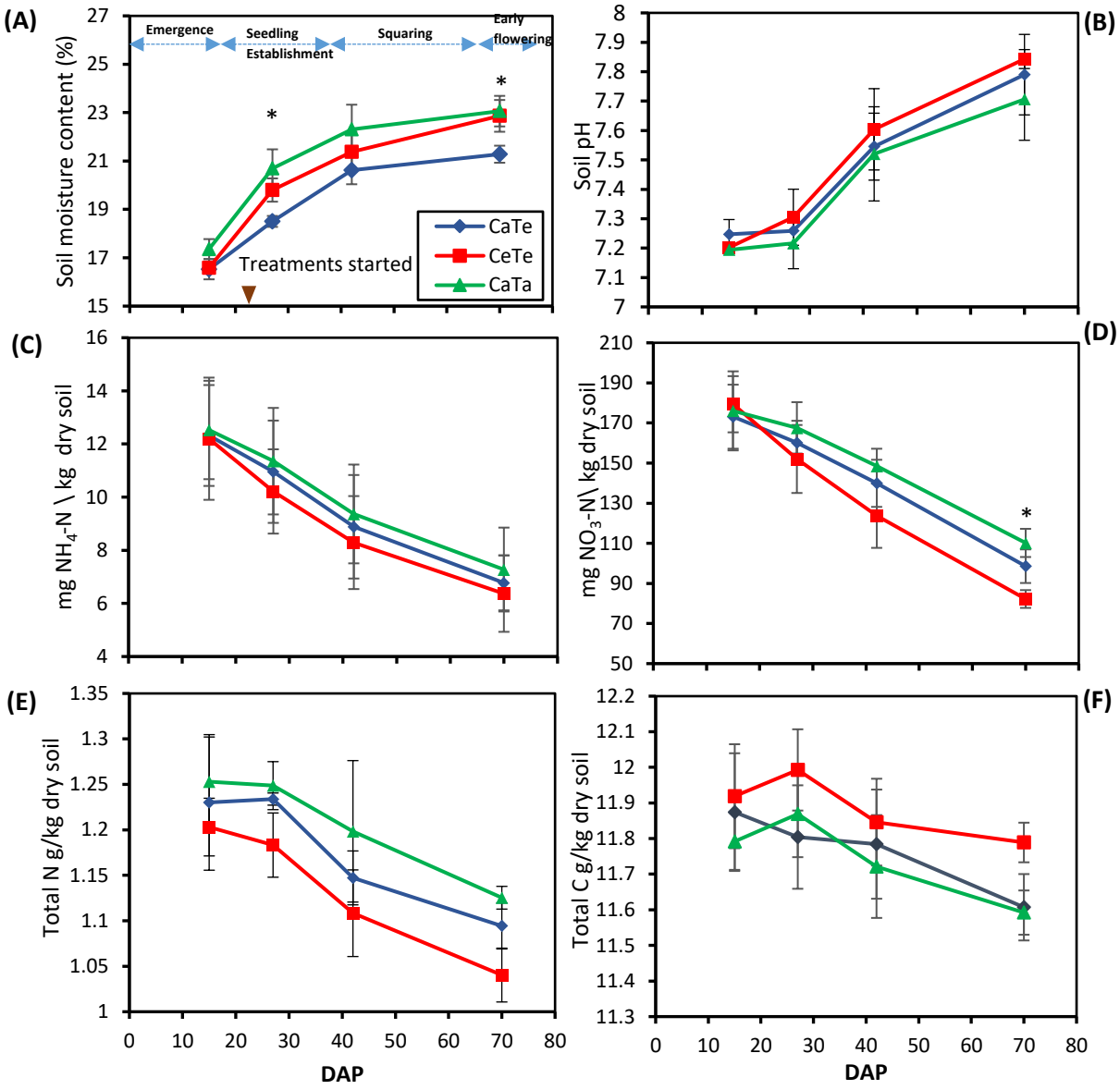

Figure 3.3 Changes in soil physicochemical properties under climate treatments during the experiment. (**A**) Soil moisture, (**B**) soil pH, (**C**) soil NH_4^+, (**D**) soil NO_3^-, (**E**) soil total N, and (**F**) soil total C. The asterisk indicates significant difference between different treatments (P<0.05). Values represent mean ± SE (n=10) of each treatment. C_aT_a = Ambient CO_2 and temperature, C_aT_e = Ambient CO_2 and elevated temperature, C_eT_e = Elevated CO_2 and elevated temperature. DAP = Day after planting. NH_4^+ = Ammonium, NO_3^- = Nitrate, N = Nitrogen, C = Carbon.

Table 3.1 Repeated measures ANOVA for the effects of climate change factors and cotton developmental stage on soil physicochemical properties. Bold values indicate a significant difference at $P<0.05$. T_e = Elevated temperature, C_eT_e = Elevated CO_2 and elevated temperature. NH_4^+ = Ammonium, NO_3^- = Nitrate, N = Nitrogen, C = Carbon.

Factor	Soil moisture	pH	NH_4^+	NO_3^-	Total C	Total N
T_e	**0.001**	0.31	0.52	0.12	0.593	0.45
C_eT_e	0.724	0.262	0.34	**<0.001**	0.201	0.32
Time (T)	**<0.001**	**0.002**	**0.004**	**<0.001**	**<0.001**	**0.001**
T_e x T	0.105	0.08	0.07	0.41	0.312	0.074
C_eT_e x T	0.496	0.09	0.21	**0.035**	**0.004**	0.08

3.3.3 Potential nitrification rate

Potential nitrification rate (PNR) varied from 0.81 to 0.95 mg N/kg dry soil/h across all treatments. PNR increased by approximately 9.8% for C_aT_a and C_aT_e plots after 70 DAP corresponding to the early flowering stage. Under C_eT_e treatment, PNR had increased by approximately 13% at the early flowering stage (70 DAP). One-way ANOVA showed that there were no significant differences in PNR between C_aT_a and C_aT_e plots at each developmental stage. In contrast, PNR of samples collected from C_eT_e plots were significantly higher when compared to those of C_aT_e at the early flowering stage (70 DAP) whereas no significant difference was observed at the other development stages (**Figure 3.4**). Repeated measure ANOVA indicated significant effects of crop developmental stage, C_eT_e and its interaction with crop developmental stage on PNR ($P<0.001$, $P=0.005$ and $P=0.002$, respectively; **Table 3.2**). In contrast, C_aT_e and its interaction with time did not significantly affect PNR ($P=0.063$ and $P=0.061$, respectively) (**Table 3.2**).

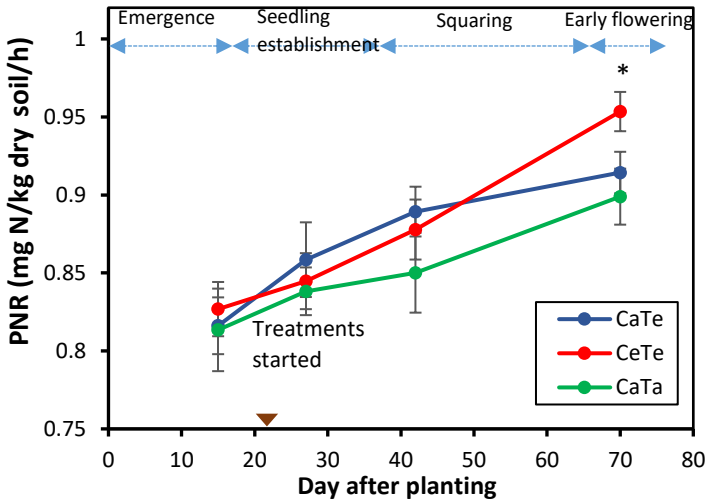

Figure 3.4 Changes in potential nitrification rate (PNR) under climate treatments during the experiment. The asterisk indicates significant difference between different treatments (P<0.05). Values represent mean ± SE (n=10) of each treatment. C_aT_a = Ambient CO_2 and temperature, C_aT_e = Ambient CO_2 and elevated temperature, C_eT_e = Elevated CO_2 and elevated temperature.

3.3.4 Ammonia-oxidizer communities

3.3.4.1 The AOB and AOA *amoA* gene abundance

The AOB *amoA* gene abundance varied from 6.06 x 10^6 to 7.98 x 10^6 copies/g dry soil, whereas AOA *amoA* gene abundance was approximately 10-fold higher, ranging from 7.06 x 10^7 to 8.43 x 10^7 copies/g dry soil across all treatments. There were upward trends for both AOB and AOA *amoA* gene copy number throughout the experiment. In particular, the abundance of AOB *amoA* genes increased by 13% and 22% for samples collected from C_aT_a and C_aT_e treatments, respectively after 70 DAP; however, no significant difference was observed at every developmental stage (**Figure 3.5**). Under C_eT_e treatment, the AOB *amoA* gene abundance increased by 29% after 70 DAP and there was a significant treatment effect on AOB *amoA* gene copy number at the early flowering

stage (70 DAP) (**Figure 3.5**). Repeated measure ANOVA showed crop growth significantly affected AOB abundance (P<0.001). T_e and T_e x time had no significant effect on AOB abundance (P=0.054 and P=0.087, respectively) whereas C_eT_e and its interaction with time had significant effects on AOB abundance (P=0.004 and P=0.008, respectively) (**Table 3.2**).

The AOA *amoA* gene abundance also increased during the experiment. It increased by approximately 9.9% for the ambient condition after 70 DAP. Under C_aT_e and C_eT_e treatments, AOA abundance increased approximately 15% and 19% after 70 DAP, respectively. At the early flowering stage (70 DAP), there were significant treatment effects on the abundance of AOA community between C_aT_e and C_eT_e; C_aT_a and C_aT_e (P= 0.009 and P=0.002, respectively) (**Figure 3.5**). Repeated measure ANOVA indicated significant effect of crop developmental stage on AOA abundance (P<0.001). T_e and T_e x time had significant effects on AOA abundance (P=0.023 and P=0.012, respectively). C_eT_e and C_eT_e x time also had significant effects on AOA abundance (P=0.003 and P=0.002) (**Table 3.2**).

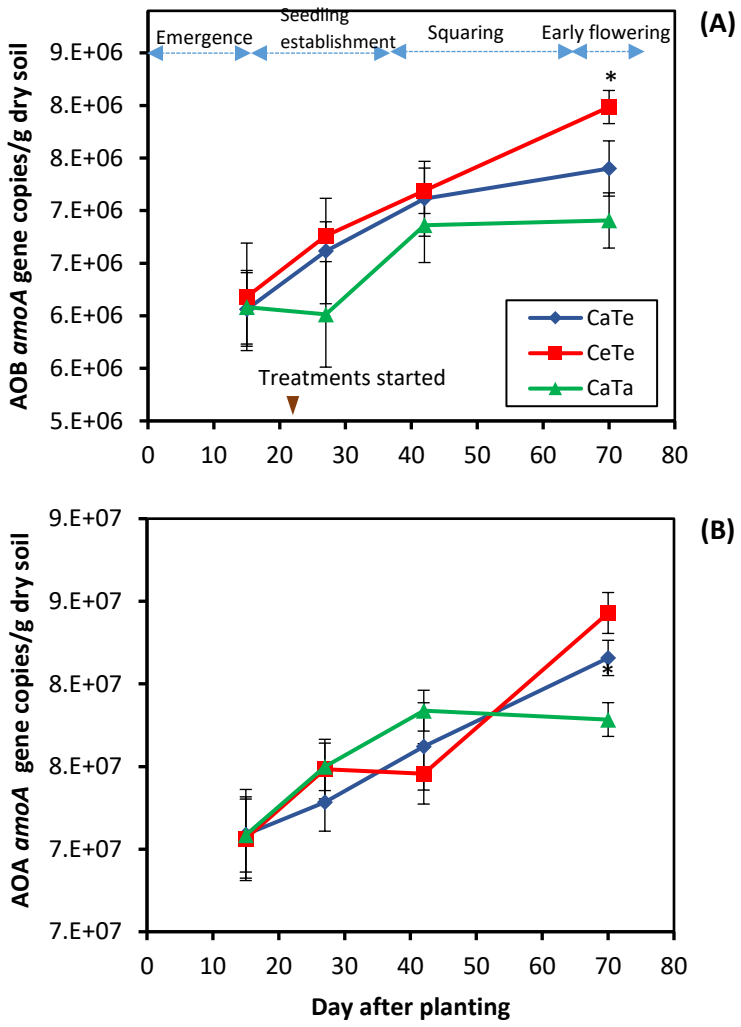

Figure 3.5 Changes in the AOB and AOA *amoA* gene abundance under climate treatments during the experiment (**A**) AOB *amoA* gene, (**B**) AOA *amoA* gene. The asterisk indicates significant difference between different treatments (P<0.05). Values represent mean ± SE (n=10) of each treatment. C_aT_a = Ambient CO_2 and temperature, C_aT_e = Ambient CO_2 and elevated temperature, C_eT_e = Elevated CO_2 and elevated temperature. AOB = Ammonia-oxidizing bacteria, AOA = Ammonia-oxidizing archaea.

Table 3.2 Repeated measures ANOVA for the effects of climate change factors and cotton developmental stage on PNR, AOB and AOA abundance. Bold values indicate a significant effect at $P<0.05$. T_e = Elevated temperature; C_eT_e = Elevated CO_2 and elevated temperature; T = Cotton growth stage. AOB = Ammonia-oxidizing bacteria, AOA = Ammonia-oxidizing archaea. PNR = Potential nitrification rate.

Factor	PNR	AOB abundance	AOA abundance
T_e	0.063	0.054	**0.023**
C_eT_e	**0.005**	**0.004**	**0.003**
T	**<0.001**	**<0.001**	**<0.001**
T_e x T	0.061	0.087	**0.012**
C_eT_e x T	**0.002**	**0.008**	**0.002**

3.3.4.2 The AOB and AOA community structures

The analysis of terminal restriction fragment length polymorphism (TRFLP) indicated four and eight different TRFs for AOB and AOA *amoA* genes, respectively. The AOB community has three dominant TRFs including TRF-55, 149 and 251 (**Figure 3.6A**). Relative abundance of TRFs have shown that TRF-74, 243 and 251 were dominant in the AOA community (**Figure 3.6B**). Under C_aT_a and C_aT_e treatments, the AOB communities marginally changed during the duration of the experiment. In contrast, under C_eT_e treatment, the relative abundance of TRF-149 and 251 significantly increased ($P<0.001$) while that of TRF-55 significantly decreased ($P=0.003$) at the early flowering stage (70 DAP). In terms of AOA community structure, no change was observed at seedling establishment (27 DAP) and early squaring stage (41 DAP) across all treatments. However, the relative abundance of TRF-54 and 91 significantly increased ($P<0.001$ and

P=0.009, respectively), whereas that of TRF-74 significantly decreased in response to C_aT_e and C_eT_e treatments at the early flowering stage (70 DAP) (*P*=0.01) (**Figure 3.6**).

The phylogenetic affiliation of some TRFs could be assigned by searching identical TRFs from the study by Hu *et al*., (2015) as mentioned in Table 2.2 in Chapter 2 (section 2.3.3.2). For the AOB community, TRF-149 and 229 were placed within the *Nitrosospira* cluster. Regarding the AOA community, TRF-74, 150 and 212 belonged to the *Nitrososphaera* cluster whereas TRF-54 and 198 were in the *Nitrosopumilus* and the *Nitrosotalea* clusters, respectively. These phylogenetic assignments combined with relative abundance of TRFs generated from TRFLP profile indicated that *Nitrosospira* significantly responded to C_eT_e while phylotypes of *Nitrosopumilus* were significantly affected by both C_aT_e and C_eT_e.

Principal coordinates analysis (PCO) of the AOB community structure indicated a strong impact of C_eT_e on the AOB community at the early flowering stage (70 DAP), with two first axes explaining 94.6% of variation in TRFLP profile (**Figure 3.7A**). Changes in the AOA community structure were also analysed by PCO in which PCO1 and PCO2 explained 84.3 % and 5.8% of the variation in AOA TRFLP profile, respectively. Data indicated that both T_e and C_eT_e had strong effects on AOA community structure at the early flowering stage (70 DAP) (**Figure 3.7B**). PERMANOVA tests showed cotton growth stages significantly affected both the AOB and AOA community structure (*P*=0.001) (**Tables 3.3** & **3.4**). T_e and T_e x growth stage significantly impacted AOA community structure (*P*=0.001) (**Table 3.4**). In contrast, C_eT_e and C_eT_e x growth stage significantly affected both AOB and AOA community structure (*P*=0.001) (**Tables 3.3 & 3.4**).

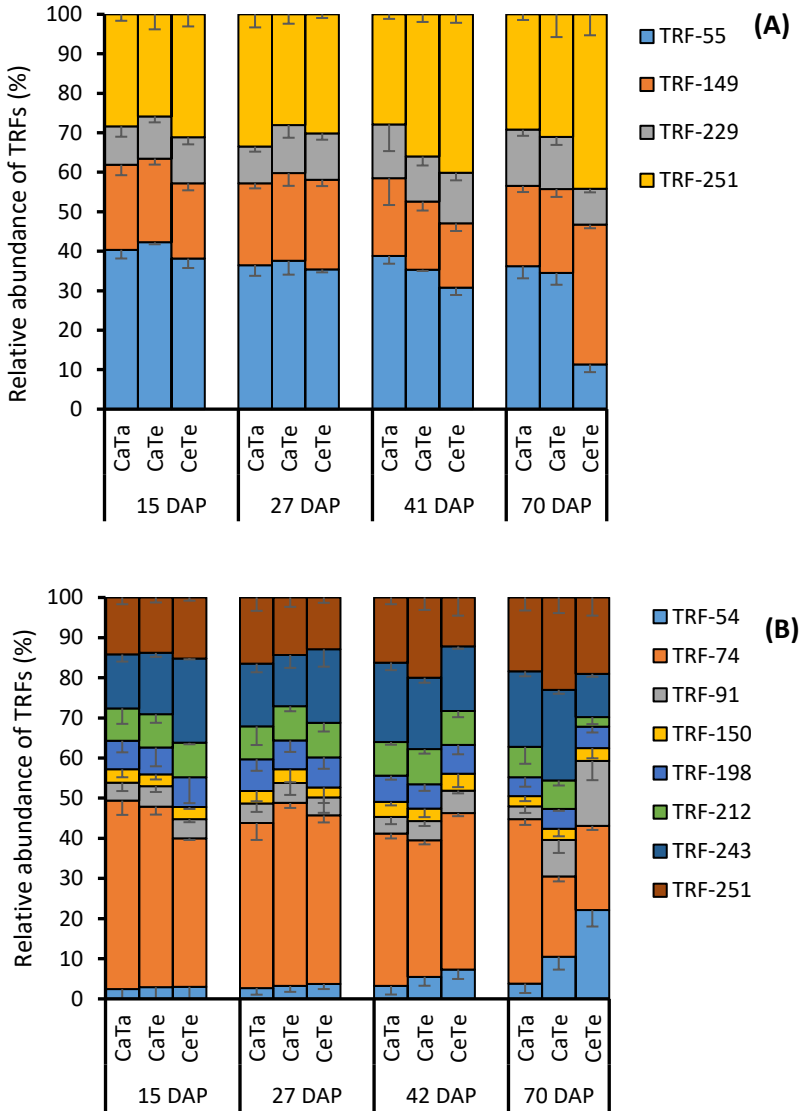

Figure 3.6 Terminal restriction fragment length polymorphism (TRFLP) fingerprints of *amoA* gene fragments under climate treatments. (**A**) TRFLP fingerprint of AOB *amoA* gene, (**B**) TRFLP fingerprint of AOA *amoA* gene. Values represent mean ± SE (n=10) of each treatment. C_aT_a = Ambient CO_2 and temperature, C_aT_e = Ambient CO_2 and elevated temperature, C_eT_e = Elevated CO_2 and elevated temperature. DAP = Day after planting. AOB = Ammonia-oxidizing bacteria, AOA = Ammonia-oxidizing archaea.

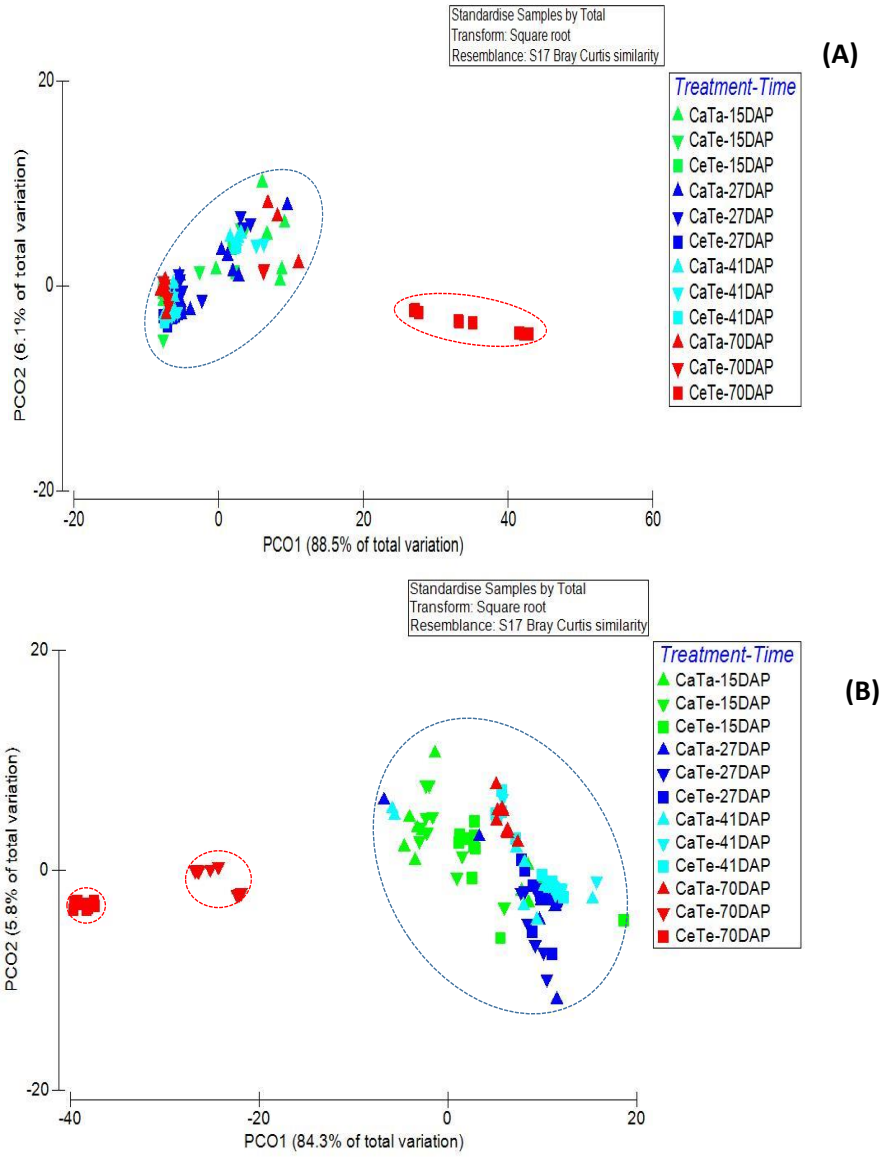

Figure 3.7 Principal coordinates analysis (PCO) derived from the Bray-Curtis dissimilarity matrices showing differences in (**A**) AOB and (**B**) AOA community structures under climate treatments at different developmental stages including emergence (15 DAP), seedling establishment (27 DAP), the early squaring stage (41 DAP) and early flowering stage (70 DAP). C_aT_a = Ambient CO_2 and temperature, C_aT_e = Ambient CO_2 and elevated temperature, and C_eT_e = Elevated CO_2 and elevated temperature.

Table 3.3 Outputs of PERMANOVA test for the treatment effects and growth stages on AOB community structure, using Type III sums of squares based 999 permutations of residuals under a reduced model. Significant effects are in bold (P<0.05). T_e = Elevated temperature, C_eT_e = Elevated CO_2 and elevated temperature, T = Cotton growth stage. SS = The sum of square, MS = The mean sum of squares, F = The F-value, P = The P-value.

Analysis	SS	MS	*F*	*P*
T_e	166.9	166.9	3.0099	0.064
C_eT_e	1509.5	1509.5	44.559	**0.001**
T	3945.6	1315.2	9.9109	**0.001**
T_e x T	175.63	58.545	1.0572	0.375
C_eT_e x T	6156.6	2052.2	60.578	**0.001**

Table 3.4 Outputs of PERMANOVA test for the treatment effects and growth stages on AOA community structure, using Type III sums of squares based 999 permutations of residuals under a reduced model. Significant effects are in bold (P<0.05). T_e = Elevated temperature, C_eT_e = Elevated CO_2 and elevated temperature, T = Cotton growth stage. SS = The sum of square, MS = The mean sum of squares, F = The F-value, P = The P-value.

Analysis	SS	MS	*F*	*P*
T_e	974.61	974.61	19.586	**0.001**
C_eT_e	342.9	342.9	11.174	**0.001**
T	16188	5395.9	37.359	**0.001**
T_e x T	4441.9	1480.6	29.756	**0.001**
C_eT_e x T	1498.5	499.52	16.278	**0.001**

3.3.5 Drivers of soil processes rates

The relationship between AOB and AOA abundance, potential nitrification rate (PNR) and soil physicochemical properties were examined by Spearman's rank correlation analysis. There were positive correlations between PNR and the abundance of AOB and AOA, which were statistically significant (r_S=0.81, *P*<0.001 and r_S=0.507, *P*<0.001, respectively). AOB abundance and PNR were significantly correlated with soil pH. Also, both the abundance of AOB, AOA and PNR were significantly correlated with total C (**Table 3.5**). The relationship between AOB, AOA structure and PNR was evaluated. My results indicated that both AOB and AOA structure had significant correlations with PNR (*P*=0.001) (**Table 3.6**). This was further confirmed by linear regression between PNR and AOB and AOA communities (**Figure 3.8**). All analyses suggested that AOB community were more strongly linked to PNR than AOA community.

Table 3.5 Correlation coefficients of soil physicochemical properties, potential nitrification rate (PNR) and the abundance of AOB and AOA communities. Significant correlations at *P*<0.01 (**) and *P*<0.05 (*) are in bold. AOB = Ammonia-oxidizing bacteria, AOA = Ammonia-oxidizing archaea, NH_4^+ = Ammonium, NO_3^- = Nitrate, Total C = Total carbon, Total N = Total nitrogen.

	Soil moisture	pH	NH_4^+	NO_3^-	Total C	Total N	PNR
AOA	0.179	0.359	-0.326	-0.353	**-0.502***	-0.323	**0.507***
AOB	0.266	**0.590****	-0.266	-0.267	**-0.413***	-0.421	**0.81****
PNR	0.321	**0.685****	-0.467	-0.127	**-0.327***	-0.265	-

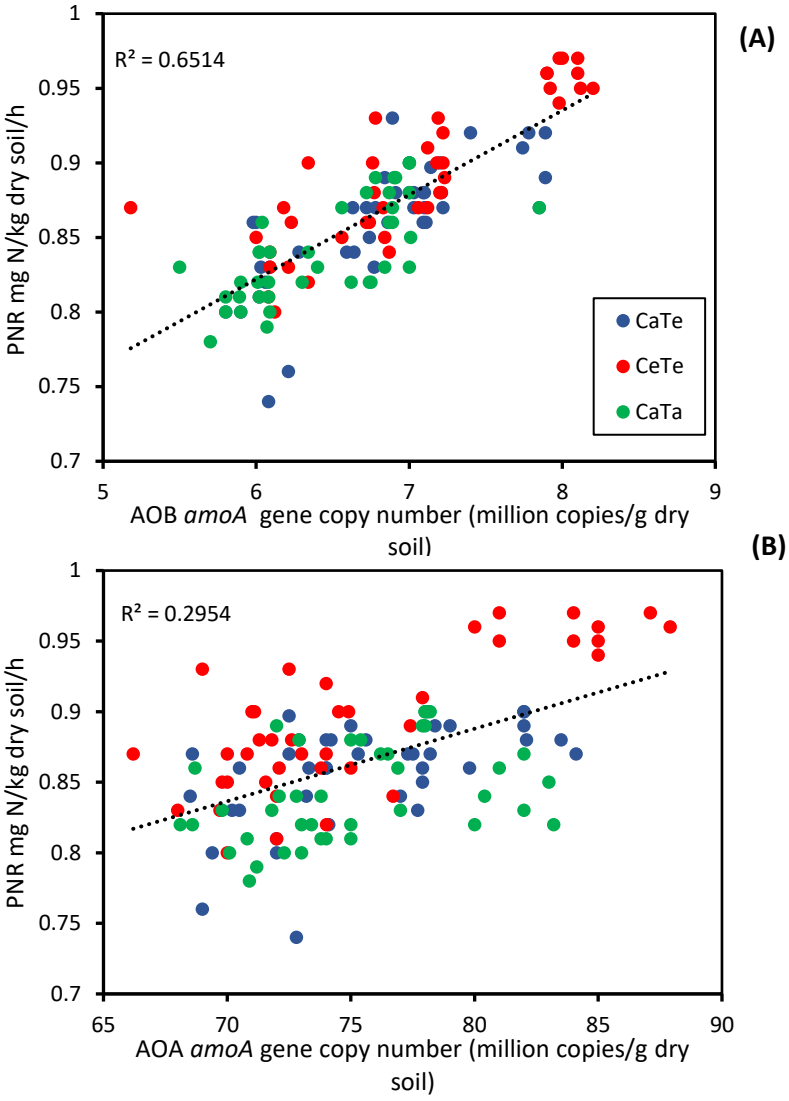

Figure 3.8 The relationship between potential nitrification rate (PNR) and the abundance of (**A**) AOB and (**B**) AOA. C_aT_a = Ambient CO_2 and temperature, C_aT_e = Ambient CO_2 and elevated temperature, C_eT_e = Elevated CO_2 and elevated temperature. AOB = Ammonia-oxidizing bacteria, AOA= Ammonia-oxidizing archaea. PNR = Potential nitrification rate.

Table 3.6 The variation explained, the Spearman rank correlation coefficient (r_S), and the *P* values of correlations between potential nitrification rate (PNR), and (**a & b**) AOB and AOA structures, as summarized by the top five principal coordinates. Significant effects are in bold (*P*<0.05). AOB = Ammonia-oxidizing bacteria, AOA = Ammonia-oxidizing archaea.

Statistic	**PCO1**	**PCO2**	**PCO3**	**PCO4**	**PCO5**
(a) AOB					
Variation explained (%)	88.5	6.1	3.31	2.02	1.2
PNR					
r_S	**-0.511**	**-0.288**	0.039	0.176	0.121
P	**<0.001**	**0.001**	0.676	0.055	0.186
(b) AOA					
Variation explained (%)	84.29	5.84	4.33	3.5	1.75
PNR					
r_S	**-0.43**	-0.148	-0.046	0.079	**0.274**
P	**0.001**	0.106	0.622	0.072	**0.002**

3.4 Discussion

In this study, by simulating higher temperature alone (T_e) and in combination with 500 ppm CO_2 concentration (C_eT_e) in field-based environmentally-controlled chambers, the

climate responses of cotton soil physicochemical properties, potential nitrification kinetics and ammonia-oxidizing communities were elucidated. The temperature inside the chambers was expected to be 2-4^{o}C warmer than outside plots; however, the real average temperature inside the chamber was approximately 1.1^{o}C higher than the ambient plot (C_aT_a). My results show that nitrification rates and ammonia-oxidizer community structure and abundance in response to climate treatments depended on the plant growth stage. In particular, only nitrification rate and ammonia-oxidizers responded to climate treatments when plants reached the early flowering stage (70 DAP). Elevated air temperatures alone did not significantly change potential nitrification rates, but increased AOA abundance and changed AOA community structure. When comparing the chambers C_aT_e and C_eT_e, both AOB and AOA abundance and structure responded to combined elevated CO_2 and temperature. Also, C_eT_e increased the potential nitrification rate. There was a significant correlation between potential nitrification rate and the abundance and structure of ammonia-oxidizers in response to C_eT_e. Overall, my findings highlight the significant effect of combined elevated CO_2 and temperature on nitrification kinetics and ammonia-oxidizing communities in a field study on irrigated cotton crops.

3.4.1 Effects of elevated temperature alone and in combination with elevated CO_2 on soil properties, AOB and AOA communities, and potential nitrification rate (PNR)

T_e treatment did not affect soil pH, NH_4^+ and NO_3^- availability, total C or total N. The AOB abundance and community structure and PNR were not impacted by T_e while only AOA abundance and community structure responded significantly. The results contrasted with my hypothesis that T_e would influence soil processes and microbes. These results can be explained by the minimal changes in soil moisture during the experiment, a key factor affecting soil nutrient availability and microbial communities in various ecosystems (Gleeson *et al.*, 2010, Barnard *et al.*, 2013, Hu *et al.*, 2015). I observed only 2% differences in soil moisture between ambient plots and T_e plots. Such minor changes in soil moisture could be due to minor differences in air temperature (only 1.1^{o}C higher)

between T_e plots and C_aT_a plots. Other factors such as soil pH and NH_4^+ are also important for nitrification and nitrifiers (Giles *et al.*, 2012); however, they did not significantly change in response to T_e in my study, and therefore did not influence PNR and ammonia-oxidizers. This might be partly attributed to unresponsive N mineralization under T_e in the experiment although increased temperatures have been reported to stimulate organic matter degradation, resulting in increased mineralized N which would benefit both nitrifiers and plant uptake (Zhou *et al.*, 2012, Nie *et al.*, 2013, Frey *et al.*, 2013); however, I did not measure N mineralization rates. Another reason for the unresponsiveness of nitrification is that there might not been have significant changes in rhizodeposition and root exudation despite reported warming-stimulated root exudation and litter decomposition in some previous studies (Uselman *et al.*, 2000, Yin *et al.*, 2013). I did not examine plant litter and root exudation in this study, but no significant differences in cotton plant biomass between T_e chambers and ambient plots were observed (Broughton 2015, pers. comm., 20 Nov).

Interestingly, I found a significant response of AOA community abundance and structure to T_e at the early flowering stage (70 DAP). It suggested AOA response to T_e was partly related to cotton growth. The response of AOA, but not AOB to T_e in my study could be attributed to their intrinsic physiological adaptation. Studies on enriched and pure AOA strains have indicated their higher ranges of optimal temperature than AOB. Tourna *et al.* (2011) and Hatzenpichler *et al.* (2008) found that the first soil AOA isolate *Nitrososphaera viennensis* and the moderate thermophilic Candidatus *Nitrososphaera gargensis* favour 37^{o}C and 46^{o}C for their growth, respectively. In addition, the thermophilic AOA *Nitrosocaldus yellowstonii* is capable of growing up to 74^{o}C (De la Torre et al., 2008). Qin *et al.* (2014) indicated *Nitrosopumilus maritimus* SCM1, a marine AOA isolate, performs the optimal growth at 32^{o}C whereas it could not survive at 10^{o}C. Opposite to AOA, their counterparts AOB have not shown the ability to survive above 40^{o}C (Jiang & Bakken, 1999, Hatzenpichler *et al.*, 2008). Thus, approximately 1.1^{o}C warmer in my study stimulated a fraction of AOA which may prefer relatively higher temperatures for their growth. This conclusion was also supported by my finding of

increased relative abundance of a dominant AOA TRF-54 which belonged to *Nitrosopumilus* cluster under warming condition.

Although the AOA community significantly responded to T_e, I did not find significant changes in PNR. This could be explained by changes in the less active or dormant fraction, thereby resulting in little difference to overall nitrification kinetics. My findings contrasted with the study by Hu *et al.* (2016) which found a strong effect of T_e on nitrification rate and AOA community in natural dryland soils. Nitrification significantly increased in response to the simulation of 3^{o}C climate warming due to shifts in AOA community structure and abundance and stimulation of AOA metabolic activities. These contradictory results suggested response of nitrification to T_e may be ecosystem-specific and could be influenced by management practices such as irrigation and fertilizer application.

The C_eT_e treatment significantly affected soil NO_3^-. Since T_e had no effect on this parameter, it can be extrapolated that elevated CO_2 alone and in combination with T_e exhibited significant effects on soil NO_3^-. The decrease of soil NO_3^- observed under C_eT_e could be due to enhanced plant uptake and/or N immobilization. Under combined C_eT_e treatments, I found that both AOB and AOA significantly responded. Due to limitations in the number of available chambers and hence the experimental design, elevated CO_2 treatment alone could not be examined, and T_e was found to significantly affect AOA community; therefore, it was difficult to conclude whether AOA community responded to elevated CO_2. However, TRFLP showed increases in TRF-54 and 91 when comparing C_eT_e and C_aT_e, reflecting that both AOB and AOA communities significantly responded to C_eT_e, resulting in alterations in the rate of nitrification which is directly related to crop yields. The significant increase in AOB and AOA abundance as well as shifts in their structures under C_eT_e in my study could be attributed to CO_2-induced plant properties. It is believed that the responses of soil microbial communities to elevated CO_2 concentrations are mainly due to changes in plant residue inputs and root exudates (Singh *et al.*, 2010). Elevated CO_2 has been demonstrated to increase C assimilation,

thereby enhancing plant biomass (Ainsworth & Long, 2005). Cotton plant biomass under C_eT_e have been reported to increase significantly (Broughton 2015, pers. comm., 20 Nov), potentially resulting in an increase in root exudates and rhizodeposition which can stimulate soil microbial communities (Rakshit *et al.*, 2012, Jin *et al.*, 2014a). My results are in line with a rice field study by Liu *et al.* (2015), in which elevated CO_2 (500 ppm) had significant effects on AOB and AOA abundance and PNR possibly due to increased flux of rhizodeposition, root exudates, and secretions in the soil which were also the explanation for stimulated nitrification under elevated CO_2 in grassland soil studies (Zak *et al.*, 1993, Carnol *et al.*, 2002, Brown *et al.*, 2012). However, a few studies on the effect of CO_2 fertilization on ammonia-oxidizers in agricultural soils have reported opposite results. Elevated CO_2 had no significant effect on AOA and AOB abundance in the rhizosphere of maize and soybean in the study by Nelson *et al.* (2010). The soybean FACE experiment also found no significant effects of elevated CO_2 on nitrifying communities (Pereira *et al.*, 2011). Such negative effects of elevated CO_2 on nitrification were explained by the enhancement of N immobilization due to potential increases in soil C/N ratio, suggesting that climate effect on nitrifying communities may also be dependent on crop type, soil type, management practices and climatic conditions.

3.4.2 The relationship between PNR and ammonia-oxidizing communities

AOA have been generally reported to outnumber their counterparts AOB in various terrestrial ecosystems (Hu *et al.*, 2015, Yao *et al.*, 2013). In support of this, the abundance of AOA was approximately 10-fold higher than AOB in my study. Both AOB and AOA were significantly correlated with PNR, suggesting that nitrification was driven by both AOB and AOA in this field-based environmentally-controlled chamber experiment, giving more insights into the relationship between microbial communities and their functioning. Although the stronger functional extent of AOB than AOA was observed, niche differentiation between AOB and AOA was not very clear in this study. My results are similar to the study by Di *et al.* (2009) and Banning *et al.* (2015) who reported the dominance of AOB over AOA in terms of activity. One explanation could be that cotton

crop systems are often well-watered and a large amount of N fertilizers applied whereas in harsh environments such as nutrient-poor drylands AOA became functionally dominant due to their advantages over AOB such as high substrate affinity and oligotrophic lifestyles (Yao *et al.*, 2013, Hu *et al.*, 2016).

3.5 Conclusions

My results indicated that the 1.1^{o}C warming had no significant effects on soil properties, PNR and AOB community. However, AOA community structure and abundance significantly responded to T_e. In contrast, C_eT_e treatment significantly affected soil NO_3^- concentration, PNR, AOB and AOA abundance and structure. Climate responses of soil properties, nitrification rate and ammonia-oxidizers were related to cotton growth stages. Such changes in rate of nitrification process and functional communities could potentially lead to alterations in soil N availability which is directly related to cotton crop productivity.

My findings also provide novel insight into the effect of climate change on the relationship between microbial communities and their functions. These findings may allow a better prediction of the response of agroecosystems, particularly N-dependent cotton crop systems, to C_eT_e, and the development of effective N management strategies within the context of projected climate change.

Since nitrification and ammonia-oxidizing community responses depended on cotton growth stages, short-term effects of T_e and C_eT_e on these variables may not be representative of long-term responses. AOA community abundance and structure may continue to alter in response to prolonged warming, thereby potentially influencing soil nitrification rates and NO_3^- content. Under long-term C_eT_e treatment, PNR and AOB, AOA community abundance and structure are predicted to be influenced. Soil NO_3^- content may also be decreased in response to long-term C_eT_e, and thus possibly generating a shortage of soil NO_3^- for plant uptake.

Elevated CO_2 alone treatment was not included in this study as there were only four chambers. The experiment was also carried out during the late season. Plots outside chamber (C_aT_a) may not be a perfect control since there were differences in radiation between inside and outside chambers. Plants grown in chambers would be affected by reduced radiation, thereby influencing microbial activity through root proliferation and exudation. Thus, further studies with fully-factorial experimental design, real-time operation, and control chambers should be conducted to more fully assess the response of soil nitrification and ammonia-oxidizers to climate change, and subsequent consequences for soil N availability and crop yields. In addition, this experiment ended when cotton reached the early flowering stage due to a drop in air temperature in April 2015, so I could not examine the effects of changes in soil substrate availability on the response of nitrification to elevated CO_2 and temperature. Thus, future experiments should be run for the whole growing season.

CHAPTER 4 RESPONSES OF SOILS AND PLANTS TO DIFFERENT NITROGEN FERTILIZER REGIMES AFTER EXPOSURE TO EXTREME WEATHER EVENTS: FLOODING AND PROLONGED DROUGHT

4.1 Introduction

Flooding, a major abiotic stress for cropping systems, may change soil nutrient availability by affecting physical and chemical characteristics of soils (e.g. porosity, structure, pH) or the size, structure and composition of the functional microbial communities, resulting in shifts in soil process rates (Ponnamperuma, 1984, Yang *et al.*, 2016). As a consequence, N loss from the flooded soil can be significant, which in combination with other adverse effects on plants, may lead to reduced crop yields. Impacts of flooding on soil physicochemical properties including nutrient availability, and on microbial communities may also significantly impact on plant productivity in subsequent years (Meisner *et al.*, 2013b, Cavagnaro, 2016). However, little is known about the impacts of flooding on soil nitrifying and denitrifying communities and the long-lasting legacy effects following flooding on these microbes and crop yields in subsequent growing seasons.

Similarly, crop plants and soils may suffer more pro-longed drought periods due to highly variable precipitation patterns in the 21st century (IPCC, 2013). The water deficit caused by prolonged-drought periods limit crop growth and physiology, and even plant mortality, thereby significantly affecting crop productivity (Xu *et al.*, 2010, Lesk *et al.*, 2016). For soils, water deficit stress can limit substrate diffusion and labile C input due to reduced plant performance, and thus soil microbes will be subjected to resource limitations which can reduce process rates (Stark & Firestone, 1995, Voroney, 2007, Brunner *et al.*, 2015). At the same time, soil microbes exposed to drought periods would alter their rates of function due to physiological stresses, potentially changing the nature

of C and N transformation (Schimel *et al.*, 2007). When soil is dried, microbes adopt acclimatization strategies by accumulating solutes such as amino acids to decrease their internal water potential to avoid dehydration and death (Harris, 1981). However, the cost of accumulating solutes is energetically expensive (Schimel *et al.*, 2007). Thus, it raises two questions: (i) whether drought events kill soil microbes or significantly reduce microbial biomass if there is a lack of adequate resource for them to adapt and (ii) how long it will take before microbial biomass recovers when normal climatic conditions return (Schimel *et al.*, 2007).

The rate of soil nitrification and denitrification is often reduced in response to drought events (Fuchslueger *et al.*, 2014, Szukics *et al.*, 2010, Van Haren *et al.*, 2005). Although drought has been shown to alter soil nitrifying and denitrifying communities in a few studies, and consequently changing nutrient cycling in soils (Sheik *et al.*, 2011, Szukics *et al.*, 2010, Gschwendtner *et al.*, 2014), the legacy responses of these microbial communities to drought conditions is poorly understood (Griffiths & Philippot, 2013, Gschwendtner *et al.*, 2014). Re-wetting dry soils can generate a pulse of C and N mineralization due to microbial resuscitation, potentially leading to an increase in C and N loss from soil (de Vries *et al.*, 2012). Numerous studies have shown plant productivity loss due to drought stress via directly influencing plant physiology and indirectly altering soil N processes (Escalona *et al.*, 2000, Wang *et al.*, 2016, Larsen *et al.*, 2011, Hartmann & Niklaus, 2012). However, drought may also have long-lasting legacy effects on soil microbial communities through its effects on soil C and N availability or directly altering soil microbial communities (Borken & Matzner, 2009, de Vries *et al.*, 2012) which can impact on crop productivity in subsequent years. Few studies have observed legacy effects of drought stress on the abundance and community structure of soil microorganisms such as nitrifiers (*amoA*), denitrifiers (*nosZ*), and mycorrhizal fungi (Banerjee *et al.*, 2016, Cavagnaro, 2016); however, nothing is known about consequences of the legacy effects of drought on soil microbial communities on crop yields.

Nitrogen is a limiting element for plant and microbial growth, therefore N fertilizer (up to 400 kg N/ha/year) has been used to fertilize agro-ecosystems to improve plant nutrition and to achieve high yield (Rosenstock *et al.*, 2013). The average rate of N-fertilizer application for irrigated cotton farming systems in Australia was 243 kg N/ha in the season 2012-2013 to maintain high fiber quality and crop productivity (Devlin & Chang, 2015). It is critical to consider soil N availability of pre-sowing soils to determine the appropriate N-fertilizer rate to avoid N-deficiency or excess N, which can negatively affect cotton crop yields (Rochester *et al.*, 2001, Ian & Bange, 2016). Crop productivity is generally enhanced by the supply of N fertilizers (Selassie, 2015, Nkebiwe *et al.*, 2016). Thus, the appropriate dose of N-fertilizer for agricultural soils with a legacy of flooding or prolonged-drought, particularly in cotton farming systems to alleviate these impacts, and consequently improving crop yields, needs to be determined.

Responses of soil microbial communities and activities to the external N supply have been investigated in numerous studies (Song & Lin, 2014, Jannoura *et al.*, 2013, Wang *et al.*, 2015, Ding *et al.*, 2016); however, the functional microbial communities in the soils, particularly nitrifiers and denitrifiers, in response to N-fertilization vary among studies and ecosystem types (Carey *et al.*, 2016, Jin *et al.*, 2014b, Tian *et al.*, 2014, Jung *et al.*, 2011, Chen *et al.*, 2012a). Although nitrification and denitrification rates are often promoted by the addition of N-fertilizers (Zhou *et al.*, 2014, Wallenstein *et al.*, 2006b, Liu *et al.*, 2011, Wang *et al.*, 2011), the role of soil nitrifying and denitrifying communities in modulating the process rates in response to N supply is poorly understood (Geisseler & Scow, 2014). AOB and AOA co-exist in most soils, but they may respond differently to N-addition due to differences in physiologies, habitat preferences and metabolism (Webster *et al.*, 2005, Offre *et al.*, 2014). A meta-analysis from 33 studies has revealed a much stronger response of AOB than AOA to N fertilization in various ecosystems (Carey *et al.*, 2016). For example, the application of N fertilizers increased the abundance of AOB, but not AOA, in alkaline cropland (Shen *et al.*, 2008), forest (Wertz *et al.*, 2012) and grassland (Shen *et al.*, 2011) soils. In contrast, He *et al.* (2007) observed a decrease in

both AOB and AOA abundance under the long-term supply of mineral N fertilizers in an acidic agricultural soil.

Regarding denitrifying communities, contradictory responses to N addition have also been reported (Jung *et al.*, 2011, Chen *et al.*, 2012a, Jin *et al.*, 2014b, Tian *et al.*, 2014). For example, only small variation (Wolsing & Priemé, 2004) and no changes (Tian *et al.*, 2014) in denitrifying communities have been observed under N fertilizer application in agricultural and grassland soils previously, whereas Enwall *et al.* (2005) found significant changes in denitrifying communities in response to N fertilization in cropland soils. Such discrepancies may be attributed to differences in ecosystem types, climatic conditions, soil types, N-fertilizer types, the duration of N fertilization and the experimental design, and thus further studies are needed. Additionally, to my knowledge, there is no study examining the effect of N fertilizer dose on soil microbial communities in the context of legacy effects of extreme weather events. Therefore, while current climate models have predicted increased frequency and magnitude of extreme weather events, it is imperative to discern whether there are legacy effects of flooding and prolonged-drought on soil processes and microbial communities which are related to crop productivity, and to what extent N fertilization can alleviate such legacies and consequences for crop yields.

In this study, by using soils previously exposed to waterlogging and prolonged-drought periods, I examined whether legacy of extreme weather events was established, and the impacts of N fertilizer application with different rates on soil nitrification and denitrification as well as cotton plant growth and crop productivity. The outcomes of this study provided insights into the relationship between microbial community and ecosystem function. Such knowledge is needed to develop N management strategies to maintain high crop productivity for cotton farming systems if the legacy effects of extreme weather events are established prior to planting. In order to achieve these objectives, I hypothesized that (i) high rates of N fertilizer addition will be required to achieve high crop productivity if soil with a legacy effect of an extreme weather event is

established due to altered soil properties; (ii) soil microbial communities, particularly ammonia-oxidizers and denitrifying bacteria will be changed in response to N fertilizer addition since soil substrate availability is altered, and (iii) AOB and AOA will respond differently to N supply due to their differences in ecological niches.

4.2 Materials and Methods

4.2.1 Glass-house experimental setup

In July 2014, bulk soils consisted of the top soil (0-10 cm) and sub-soil (10-20 cm) were collected from the cotton field which was exposed to two events of waterlogging simulated for 120 hours by running furrow irrigation at the early and late flowering stages in January and February 2014, respectively at the Australian Cotton Research Institute (ACRI) at Narrabri (30.31^{o}S, 149.78^{o}E) in north-west New South Wales, Australia. The characteristics of regional climate, soils and annual rainfall were mentioned in Chapter 2 (section 2.2.1). The bulk soils were taken from waterlogged and control areas, and then immediately transferred to Western Sydney University (WSU).

At WSU, soils were kept in large aerated bags and stored at Hawkesbury Forest Experiment (HFE) site and soil moisture was checked regularly. Due to the low moisture content recorded (approximately 15% in January, 2015), watering was conducted twice a month from the beginning of February to the end of May, 2015. Each watering time provided an amount of water that was equal to 22 mm rainfall, which represented half of the mean monthly rainfall which cotton fields receive during the winter fallow period, to simulate natural conditions as significant N loss through leaching often occurs in the field. Soil moisture was checked every two weeks and presented in Figure 4.1. The moisture content of control and post-waterlogging soils was approximately 26% before filling pots (**Figure 4.1**).

Half of the soils collected from the control area were spread on a tarp in a shed for air-drying to simulate prolonged-drought condition for 4 months (from beginning of February to the end of May, 2015) according to an established method for drought

impacts (Meisner *et al.*, 2013a). The moisture of air-dried soils was checked every two weeks and described in Figure 4.1. The moisture content of prolonged drought-simulated soil was approximately 6.2% before filling pots (**Figure 4.1**).

In the first week of June 2015, top-soil (0-10 cm) and sub-soil (10-20 cm) were placed into plastic pots (25 x 23 x 19 cm), and then the pots were moved to glasshouse chambers (~50 m^3 each) with controlled temperature. The chamber was set up at 28^oC/17^oC (day/night) without humidity control. These conditions were to mimic annual mean climatic conditions in the field in Narrabri. There were three water treatments: Control, Post-waterlogging (Post-WL) and Post-prolonged drought (Post-PD). All pots were watered to field capacity and were allowed to settle for two days prior to application of N fertilizer. Four different urea fertilizer (46% of N) rates were applied into pots with 4 replicates, including 0 kg N ha^{-1}, 100 kg N ha^{-1}, 200 kg N ha^{-1} and 300 kg N ha^{-1}. Other nutrients such as P, K and trace elements were applied in the same amounts to all pots to mimic commonly applied nutrients for irrigated cotton in the field (see section 2.2.2 of Chapter 2). In total, there were 48 pots (3 water treatments x 4 different levels of N input x 4 replicates). Four CSIRO cultivar Sicot BRF71 seeds were sowed into each pot and no water was added until plant emergence. When plants had true leaves, they were thinned to one plant per pot. All pots were watered every 2-3 days to bring them back to field capacity. LED light was installed in the chamber to support cotton growth and development. The LED light with an intensity of 70 µmol m^{-2} s^{-1} was on daily from 6:00 am to 6:00 pm.

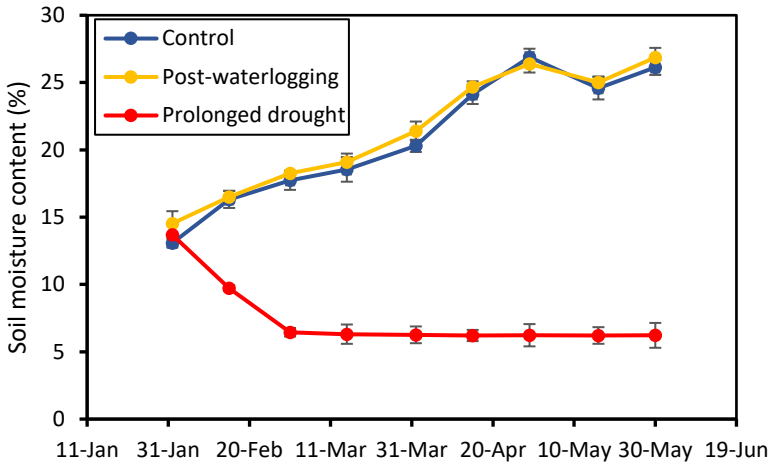

Figure 4.1 Changes in the moisture of control, post-waterlogging, and prolonged drought-simulated soils during 4 months (from the early of Feb to the end of May, 2015). Values represent mean ± SE (n=5) of each soil water treatment.

4.2.2 Soil sampling

Two soil cores of 3 cm diameter and 10 cm deep were taken from each pot at each sampling time. Soil samples were collected four times including pre-sowing (11st June, 2015 prior to N fertilizer application), the early squaring (74 DAP for control and Post-WL pots; and 89 DAP for Post-PD pots), the early flowering (95 DAP for control and Post-WL pots; and 108 DAP for Post-PD pots) and harvest (172 DAP for control and Post-WL pots; and 186 DAP for Post-PD pots). After each sampling time (except the harvest), soil holes created by coring were filled with soil from spare pots. All collected soil samples were immediately sieved through 4 mm-mesh sieve to remove plant residues, and subsequently stored at 4^{o}C for further analyses. Also, sub-samples were collected and kept at -20^{o}C for molecular processing.

4.2.3 Soil physicochemical analyses

Soil physicochemical properties including soil moisture, pH, NH_4^+, NO_3^- and total C & N were determined according to the methods described in Chapter 2 (section 2.2.5).

4.2.4 Soil process measurements

4.2.4.1 Net nitrification and N mineralization

Net nitrification and N mineralization rate (NNR and NMR) were measured based on the net amount of NO_3^- and NH_4^+ produced in soil incubated at field capacity and 25^oC over a period of 28 days (Hart *et al.*, 1994). Firstly, a 10 gram of soil was processed to determine initial concentrations of NH_4^+ and NO_3^- by adding 100 ml of 2M KCl to the soil, then shaking at room temperature for 1 hour before filtered through Whatman No. 42 filter paper. The extract was then analyzed by a SEAL AQ2 analyzer (SEAL Analytical, Maquon, WI, USA). The other 10 grams of each soil sample was placed into a 50 ml Falcon tube and the final moisture content was adjusted to 60% field capacity. Tubes were then incubated in the dark at 25^oC for 28 days and analyzed NH_4^+ and NO_3^- concentrations according to the above method. NNR and NMR were determined as differences of NO_3^- and NH_4^+ concentration between initial and incubated samples.

4.2.4.2 Potential nitrification rate

Potential nitrification rate (PNR) was determined using the chlorate inhibition method from Kandeler & Böhm (1996). The detailed method was described in Chapter 2 (section 2.2.6).

4.2.4.3 Potential denitrification rate

Potential denitrification rate (PDR) was measured according to the acetylene inhibition method (Smith & Tiedje, 1979). A 10 g of moist sieved soil was placed into 160 ml Wheaton serum bottle which then was added 20 ml of the solution containing 50 µg NO_3^- and 300 µg C g $soil^{-1}$ to make a slurry. The bottle was sealed with a butyl rubber stopper and aluminum cap with center opening (Sigma-Aldrich, USA). The anaerobic environment was created by flushing N_2 gas for 20 minutes. A headspace volume of 10% was removed and then replaced by pure acetylene. The bottle was then placed in a rotary shaking machine set at 150 rpm and incubated at 25^oC for 5 hours. A 5 ml gas sample

was collected every hour, and then injected into evacuated vials (Agilent Technologies, USA) sealed with a butyl rubber stopper and aluminum cap (Sigma-Aldrich, USA). Gas sample was analyzed for N_2O production using 7890A gas chromatography with a G1888 network headspace sampler (Agilent Technologies, USA) equipped with a micro electron capture detector (μECD) for N_2O. The calibration curve was obtained from a series of dilutions from known standard concentration (1ppm for N_2O). Standard was included every time of the sample analysis. PDR was expressed as μg N_2O-N per kg soil dry weight per hour.

4.2.5 Microbial community analyses

4.2.5.1 DNA extraction

Total soil genomic DNA was extracted using the MoBio PowerSoil DNA Isolation kit (MoBio Laboratories, Carlsbad, CA, USA) according to manufacturer's instruction. The detailed procedures were mentioned in Chapter 2 (section 2.2.7.1). Extracted DNA was kept at -20^{o}C until analysis.

4.2.5.2 Quantitative PCR

The abundances of ammonia-oxidizers and N_2O-reducing bacteria were quantified by quantitative PCR (qPCR) of bacterial and archaeal *amoA* gene (AOB and AOA) and *nosZ* gene. Two pair of primers used to amplify AOB and AOA *amoA* genes were amoA-1F/amoA-2R and CrenamoA23f/CrenamoA616r (Hallin *et al.*, 2009). PCR reaction components and thermal conditions for AOB and AOA were described in Chapter 2 (section 2.2.7.2). The copy number of N_2O-reducing bacteria was determined using the pair of primers NosZ2f/NosZr (CGCRACGGCAASAAGGTSMSSGT/CAKGTRCAKSGCRTGGCA

GAA) (Henry *et al.*, 2006). Each sample was quantified in 10 μl reaction including 5 μl GoTaq® qPCR Master Mix (2X), 20μM each primer, 0.1 μl CXR reference dye and 10 ng of template. PCR thermal conditions for *nosZ* were as follows: an initial cycle of 95^{o}C for 15 min; 6 cycles of 95^{o}C for 15 s, 65^{o}C for 30 s (with a touch down of -1^{o}C by cycle), 72^{o}C for

30 s, 80°C for 15 s; 40 cycles of 95°C for 15 s, 60°C for 15 s, to 72°C for 30 s, 80°C for 15 s; 1 cycle of 95°C for 15 s, 60°C for 15 s, to 95°C for 15 s (Henry *et al.*, 2006).

Details of standard plasmids for AOB and AOA *amoA* genes and calibration curves were described in Chapter 2 (section 2.2.7.2). Regarding *nosZ* gene, standard plasmid was constructed by cloning PCR products into the pGEM-T easy vector (Promega, Madison, USA). Calibration curves were generated by the 10-fold serial dilution of plasmids. Melt curve analyses from 65 to 95°C were conducted following each assay to verify the specificity of the amplification products. PCR efficiency values for the AOB and AOA *amoA*, and *nosZ* gene abundance were in the ranges of 88-97%, 83-94%, and 85-96%, respectively.

4.2.5.3 Terminal restriction fragment length polymorphism

The community structures of AOA and AOB and N_2O-reducing bacteria were determined by using TRFLP. The fragments of AOA and AOB *amoA* genes were amplified using fluorescently labelled primers FAM-CrenamoA23f/CrenamoA616r and VIC-amoA-1F/amoA-2R respectively. Regarding N_2O-reducing bacteria, the pair of primers 1211F/1917R (CGYTGTTCMTCGACAGCCA/CATGTGCAGNGCRTGGCAGAA) labelled with the fluorescent dye VIC was used to amplify the fragments of *nosZ* (Scala & Kerkhof, 1998). The details of primer sequences, PCR reaction components and thermal conditions for AOB and AOA were described in Chapter 2 (section 2.2.7.3). Regarding *nosZ* gene, the concentration of each component for PCR amplification was similar to that of PCR reactions for AOB and AOA *amoA* genes. PCR of *nosZ* were performed using the cycle condition: 95°C for 5 min; 30 cycles of 95°C for 1 min, 55°C for 30 s, 72°C for 1 min; 72°C for 7 min (Scala & Kerkof, 1998). PCR amplicons were then visualized on 1% (w/v) agarose gel under UV radiation to check whether successful amplification was achieved.

PCR products were purified and then checked for concentration according to methods described in Chapter 2 (section 2.2.7.3). The concentration of purified DNA ranged from

56 to 78, 50 to 62 and 60 to 104 ng/µl for AOB, AOA *amoA*, and *nosZ*, respectively. The ratio of $A_{260/280}$ and $A_{260/230}$ for all genes varied in the range of 1.8-2.1 and 0.76-1.83, respectively.

Purified PCR products were then subjected to digestion by commercial restriction enzymes. AOB and AOA gene fragments were digested by MspI and HpyCH4V, respectively (NewEngland BioLabs, USA). The restriction enzyme MspI was also used to digest the PCR products of *nosZ*. The detailed components and conditions of enzyme digestion were described in Chapter 2 (section 2.2.7.3). The procedures to analyze TRFLP profile after resolving on an ABI PRISM 3500 Genetic analyzer (Applied Biosystems, CA, USA) were also described in detailed in Chapter 2 (section 2.2.7.3).

4.2.6 Plant growth and productivity measurements

Plant height and number of nodes were determined at the early squaring, early flowering stages, and harvest time. Cotton plants were harvested at 176 DAP for plants grown on control and Post-WL soils, and at 195 DAP for plants grown on Post-PD soils. Harvested plants were separated into vegetative organs including leaf, stem, and root. Leaf number was counted. Total plant leaf area (cm^2) was determined by a portable leaf area meter (LI-3100A, LI-COR, Lincoln, NE, USA). Leaf, stem, and root were oven-dried at 70^oC for 72h and then weighted for dry mass. The number of bolls were counted and the physical boll size was determined by using calipers. The total dry mass and seed cotton yields were also determined. Total C and N content of leaf, stem, root and cotton seed were analyzed by LECO macro-CN analyzer (LECO, MI, USA).

4.2.7 Statistical analysis

One-way ANOVA with Tukey's HSD was applied to test the legacy effect of soil water treatments on soil physicochemical properties, soil process rates, and molecular measures at the pre-planting stage.

Two-way ANOVA with Tukey's HSD was used to determine the effects of N-addition and soil water treatment legacy on soil chemical properties, soil process rates and molecular measures at each developmental stage of cotton plants.

Two-way repeated measures ANOVA with N-addition and soil water treatment legacy as between-subject factors and sampling time as within-subject variable was applied to test each treatment effect and their interaction effects on soil physicochemical properties, soil process rates and molecular measurements.

Spearman rank correlation analysis was conducted to examine the relationship between variables including soil physicochemical properties, plant measurements, the copy number of AOB, AOA *amoA* genes, *nosZ* genes, soil process rates and the community structure of total bacteria, AOB, AOA and NO_2-reducing bacteria. The copy number of quantified genes was log-transformed prior to statistical analysis to meet normality assumptions. $P<0.05$ was considered to be statistically significant. All these tests were carried out using SPSS 22 (IBM, Armonk, NY, USA).

The PCO analysis was conducted to visualize the Bray-Curtis dissimilarity matrices based on the relative abundance of AOB, AOA and NO_2-reducing bacteria using Primer v6 (Primer-E Ltd, Plymouth, UK), following PERMANOVA to examine the significance of Bray-Curtis dissimilarity.

4.3 Results

4.3.1 Effects of waterlogging and prolonged-drought on soil conditions prior to planting: soil physicochemical properties, processes, and microbial communities

Soil properties were examined before planting. All pots were watered to field capacity two days prior to the sampling date. Before planting, soil moisture of all pots was not much different. In particular, it was approximately 24% for all pots (**Table 4.1**). The pH of three different soil treatments was also quite similar. Soil pH of control and Post-PD pots were 8.0, whereas that of Post-WL soils was 7.92. Soil nutrients including inorganic N,

total C and N were significantly lower in Post-PD soils (P<0.001) (**Table 4.1**). One-way ANOVA analysis showed no or marginally significant difference in the concentration of NH_4^+, total N and C between control and Post-WL soils (P=0.07, P=0.065 and P=0.13, respectively); however, NO_3^- profile of Post-WL soils was significantly different from that of control soils (P=0.01) (**Table 4.1**).

Soil functions including potential nitrification rate (PNR), potential denitrification rate (PDR), net nitrification and N mineralization rate (NNR and NMR) were measured for all soils before planting. PNR of control and Post-WL soils were approximately 0.53 and 0.49 mg N/kg soil dwt/h, respectively. One-way ANOVA showed no significant difference of PNR between control and Post-WL soils (P=0.065) (**Table 4.2**). In contrast, significantly lower PNR was observed for Post-PD soils (P=0.007) (**Table 4.2**). Potential denitrification rate (PDR) of Post-WL soils was higher than that of control soils (P=0.02). Similarly, PDR of control and Post-PD soils were significantly different (P=0.011) (**Table 4.2**). Net nitrification and N mineralization rates (NNR and NMR) were also lower in Post-PD soils (P=0.021). In contrast, no significant differences in NNR and NMR were observed between control and Post-WL soils (P=0.067) (**Table 4.2**).

Table 4.1 Soil physicochemical properties before planting. Data are presented as means of four replicates ± SE of each treatment. Different letters within the same column indicate significant differences between control and Post-WL soils; and control and Post-PD soils. Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought, NH_4^+ = Ammonium, NO_3^- = Nitrate, Total N = Total nitrogen, Total C = Total carbon.

Treatment	Soil moisture	pH	NH_4^+ (mg/kg)	NO_3^- (mg/kg)	Total N (g/kg)	Total C (g/kg)
Control	24.23 ± 0.71a	8.00 ± 0.09a	8.47 ± 0.27a	24.84 ±0.69a	0.52 ± 0.01a	8.18 ± 0.12a
Post-WL	24.86 ± 0.49a	7.92 ± 0.08a	8.41 ± 0.12a	17. 54 ±0.71b	0.50 ± 0.01a	8.02 ± 0.11a
Post-PD	24.35 ± 0.26a	8.02 ± 0.08a	5.69 ± 0.11b	17.13 ±0.55b	0.41 ± 0.008b	7.06 ± 0.08b

Table 4.2 Soil potential and net nitrification rate (PNR and NNR), potential denitrification rate (PDR), net mineralization rate (NMR) before planting. Data are presented as means of four replicates ± SE of each treatment. Different letters within the same column indicate significant differences between control and Post-WL soils; and control and Post-PD soils. Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought.

Treatment	PNR (mg NO_2-N/kg dry soil /h)	NNR (mg NO_3-N/kg dry soil/day)	PDR (µg N_2O-N/g dry soil/h)	NMR (mg N /kg dry soil /day)
Control	0.53 ± 0.01a	0.18 ± 0.02a	0.14 ± 0.01a	0.11 ± 0.01a
Post-WL	0.49 ± 0.04a	0.16 ± 0.01a	0.21 ± 0.02b	0.10 ± 0.01a
Post-PD	0.37 ± 0.01b	0.07 ± 0.03b	0.09 ± 0.01b	0.06 ± 0.009b

AOB, AOA *amoA* and *nosZ* gene abundances were quantified for samples collected before planting. AOB *amoA* gene abundance of Post-WL soil was lower than that of control, however, no significant difference was observed (P=0.15). In contrast, AOB *amoA* gene abundance of Post-PD soil was 7-fold lower when compared to control (P= 0.003) (**Table 4.3**). AOA *amoA* gene abundance was not significantly different between control and Post-WL soils (P=0.34), and control and Post-PD soils (P=0.13) (**Table 4.3**). In terms of *nosZ* gene abundance, the Post-WL soils had 5.4-fold higher *nosZ* gene abundance than control and they were significantly different from each other (P=0.013). The *nosZ* gene abundance of Post-PD soils was approximately 3.4-fold lower than that of control soil (P=0.021) (**Table 4.3**).

Table 4.3 AOB, AOA *amoA* and *nosZ* gene abundances before planting. Data are presented as means of four replicates ± SE of each treatment. Different letters within the same column indicate significant differences between control and Post-WL soils; and control and Post-PD soils. Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought, AOB = Ammonia-oxidizing bacteria, AOA = Ammonia-oxidizing archaea.

Treatment	AOB *amoA* (x10^6)	AOA *amoA* (x 10^8)	*nosZ* (x 10^7)
Control	6.32 ± 0.28a	1.48 ± 0.44a	1.05 ± 0.17a
Post-WL	5.84 ± 0.25a	1.29 ± 0.38a	5.67 ± 0.21b
Post-PD	0.90 ± 0.02b	1.16 ± 0.076a	0.31 ± 0.064b

Analysis of TRFLP profile generated the information of relative abundance of TRFs. The relative abundance of AOB-TRFs of control and Post-WL soils were not much different. There were four different TRFs generated for control and Post-WL soils, in which three dominant TRFs were 55, 149 and 251. The relative abundance of TRF-55 and 251 decreased in Post-WL soil; however, only marginally or no significant difference was observed (P=0.085) (**Figure 4.2**). In Post-PD soils, three dominant TRFs including 55, 149 and 251 were also obtained. The TRF-229 disappeared when compared to control samples (**Figure 4.2**). Regarding the relative abundance of AOA *amoA* fragments, there were seven different TRFs generated, and only marginally or no significant difference was observed among soil treatments (P=0.066) (**Figure 4.2**). For the relative abundance of *nosZ* fragments, seven different TRFs were also generated across all treatments, in which TRF-56, 72 and 105 were dominant. TRF-107, 214 and 332 of Post-WL soils were significantly increased (P=0.014; P=0.023; and P=0.031; respectively) when compared to control. In Post-PD soils, TRF-72 and 105 significantly decreased (P=0.01) (**Figure 4.2**).

Differences in the community structure of AOB, AOA and N_2O-reducing bacteria between soil water treatments were examined by principal coordinate analysis (PCO). The two first axes in the PCO analysis explained 99.8% of variation in the TRFLP profile of AOB.

The AOB community structure of control and Post-WL soils was separated from that of Post-PD soils **(Figure 4.3A).** In contrast, the PCO analysis with PCO1 and PCO2 explained 87.9% and 9.2% of AOA variation, respectively showed no clear separation of AOA community structure between treatments (**Figure 4.3B**). In terms of N_2O-reducing bacterial community structure, there was separation among soil water treatments in the PCO analysis in which PCO1 and PCO2 explained 77.6% and 11.1% of the variation in this bacterial community structure (**Figure 4.3C**).

PERMANOVA test indicated significant differences in the community structure of AOB between control and Post-PD soils (P=0.001). Although the separation in AOB community between control and Post-WL soils was small, PERMANOVA showed a statistically significant difference (P=0.001). In terms of N_2O-reducing bacterial community structure, there were statistically significant differences among soil water treatments (P=0.001) (**Table 4.4**).

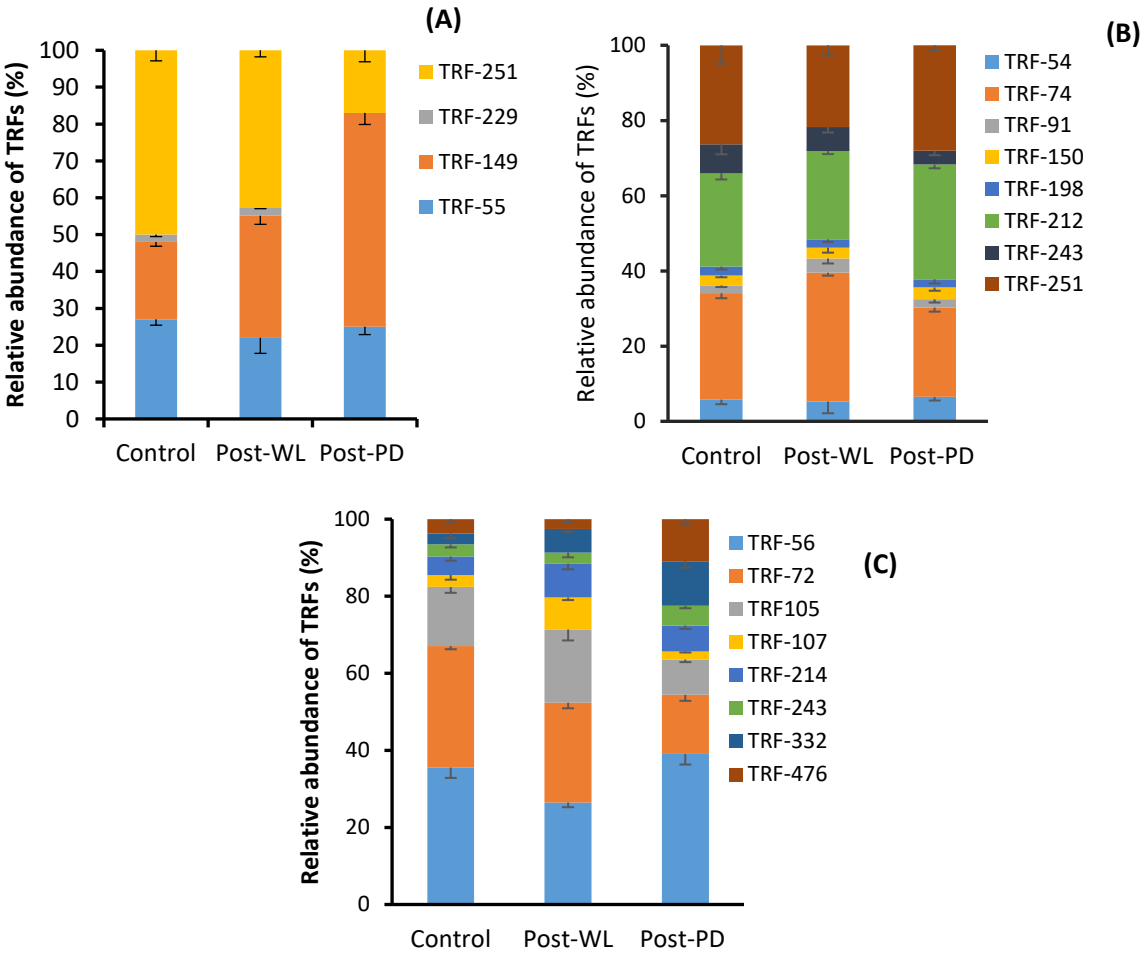

Figure 4.2 Terminal restriction fragment length polymorphism (TRFLP) fingerprints of (**A**) AOB, (**B**) AOA *amoA* gene and (**C**) *nosZ* gene before planting across all soil water treatments. Values represent mean ± SE (n=16) of each soil water treatment. AOB = Ammonia-oxidizing bacteria, AOA = Ammonia-oxidizing archaea. Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought.

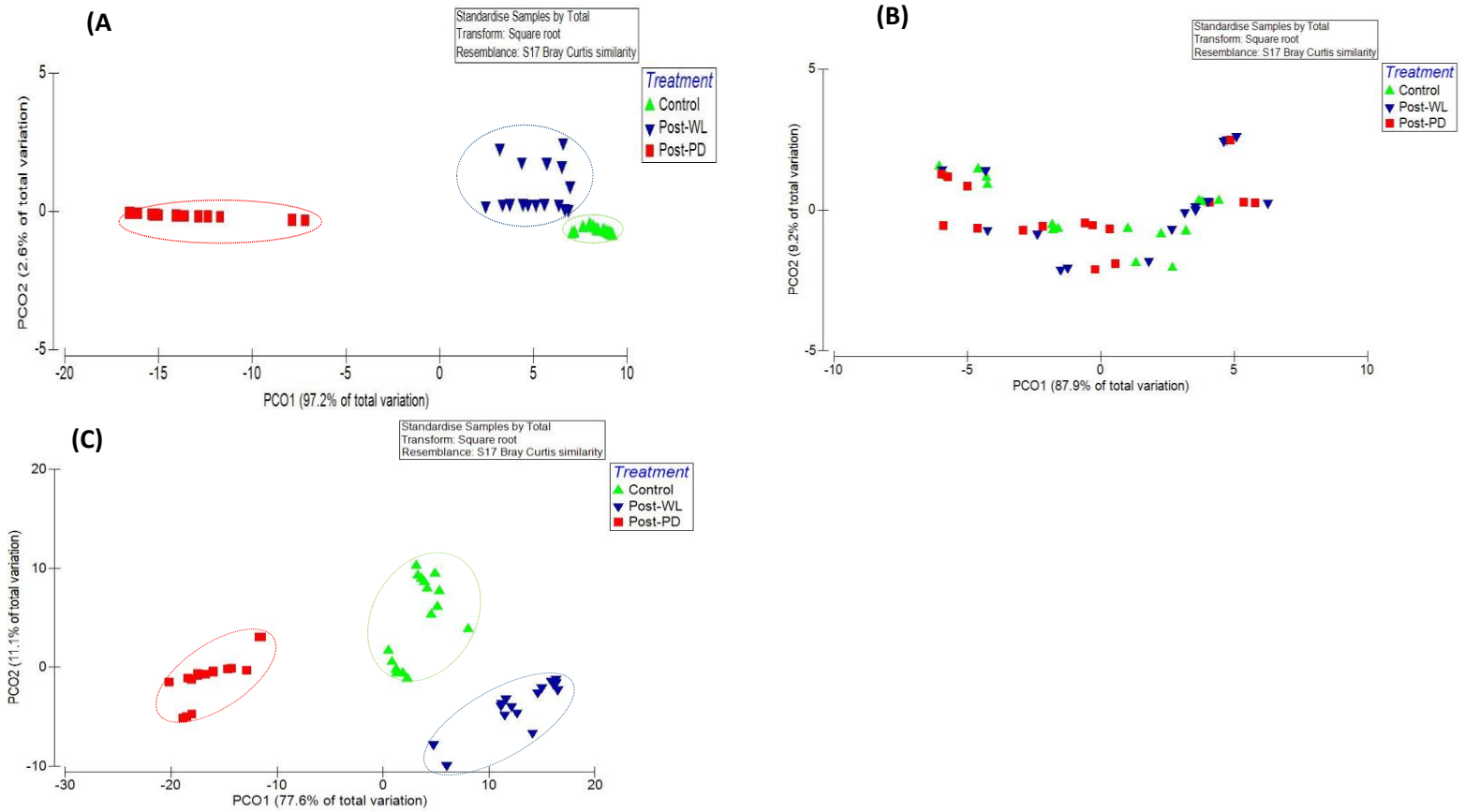

Figure 4.3 Principal coordinates analysis (PCO) derived from the Bray-Curtis dissimilarity matrices showing differences in (**A**) AOB, (**B**) AOA, and (**C**) N_2O-reducing bacterial community compositions among soil water treatments at pre-planting. Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought. AOB = Ammonia-oxidizing bacteria, AOA = Ammonia-oxidizing archaea, N_2O = Nitrous oxide.

Table 4.4 Results from PERMANOVA testing for the effects of waterlogging and prolonged drought on AOB, AOA and NO_2-reducing bacterial structures, using Type III sums of squares based 999 permutations of residual under unrestricted permutation of raw data. Significant effects are in bold (P<0.05). F = The F-value, P = The P-value. Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought. AOB = Ammonia-oxidizing bacteria, AOA = Ammonia-oxidizing archaea, N_2O = Nitrous oxide.

Analysis	*F*		*P*	
	Post-WL	**Post-PD**	**Post-WL**	**Post-PD**
AOB structure	60.592	867.52	**0.001**	**0.001**
AOA structure	1.3384	0.02259	0.223	0.964
N_2O-reducing bacterial structure	39.71	94.393	**0.001**	**0.001**

4.3.2 Effects of different N fertilizer rates on plant and soil

4.3.2.1 Plant growth and productivity

Plant height and number of nodes were determined for each developmental stage including early squaring, early flowering, and final harvest. At the early squaring stage, the height of cotton plants varied from 18 cm to 56 cm across all soil water treatments and different N-fertilizer rates. Without N-fertilizer addition, the height of cotton plants grown on Post-WL soil were significantly different from that of plants grown on control soils (P=0.023). Similarly, cotton plant heights grown on Post-PD soils were significantly smaller than plants grown on control soils (P=0.014). The height of cotton plants increased when N-fertilizer increased across all soil treatments (**Table 4.5** & **Figure 4.4**). At the rate of 100, 200 and 300 kg N/ha, there were no significant differences in plant height between control and Post-WL soils (P=0.34, P=0.45, P=0.12, respectively) (**Table 4.5**). In contrast, the height of plants grown on Post-PD soils was significantly lower than that of control soils at all N-fertilizer rates (**Table 4.5**).

At the early flowering stage, the height of plants was in the range of 29-67 cm across all soil treatments and N-fertilizer rates. The same trends were observed when compared to the early squaring stage. The only difference is that plants grown on Post-WL soils caught up to those grown on control soils at the rate of 0 kg N/ha (**Table 4.5 & Figure 4.5).**

At harvest, the plant height varied from 32 to 76 cm across all soil treatments and N-fertilizer rates. The trends observed for this sampling time point were similar to that of the early flowering stage (**Table 4.5**). In terms of plant nodes, the number were in the range of 5-12 nodes, 6-14 nodes, and 8-17 nodes for the early squaring, early flowering, and harvest, respectively. Similar to plant height, at the early squaring stage and 0 kg N/ha, number of nodes of Post-WL plants was significantly lower than control plants (P=0.013), but Post-WL plants caught up to the control plants at the early flowerings stage (P=0.092). For other rates of N fertilizer including 100, 200 and 300 kg N/ha, the same trends were observed for all sampling time points. In particular, no significant differences in the number of nodes per plant were found between control and Post-WL plants while the nodes of Post-PD were significantly lower than that of control plants (**Table 4.5**).

Two-way ANOVA indicated that there were statistically significant effects of N-addition on plant height and node (P<0.001) at each developmental stage (**Table 4.7**). The waterlogging event simulated in the previous season did not establish legacy effects on plant height and node (P=0.42 and P=0.603, respectively), whereas there were legacy effects of the prolonged-drought event on plant height and node (P<0.001). No interaction effects of soil moisture legacy and N-addition on plant height and node were found for Post-WL and Post-PD pots (P>0.05) at each developmental stage (**Table 4.7**).

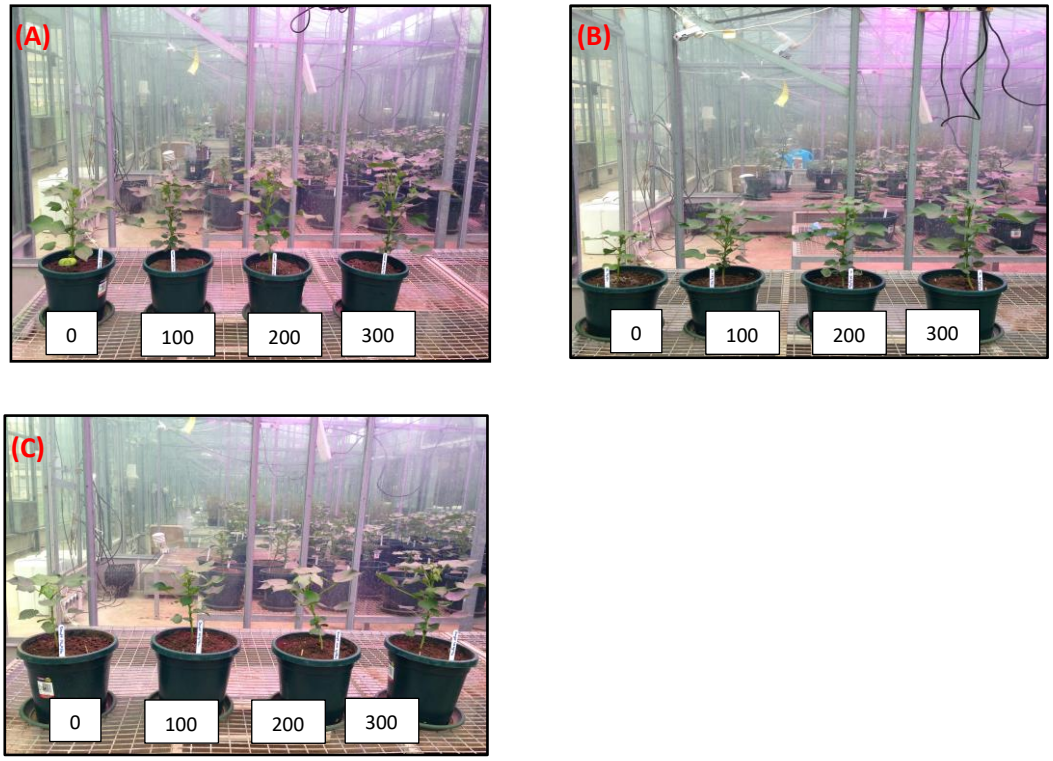

Figure 4.4 Cotton plants grown in glasshouse with different N fertilizer rates including 0, 100, 200 and 300 kg N/ha. Cotton plants grown on (**A**) control and (**B**) Post-WL soils were at the early squaring stage (74 DAP) whereas plants grown on Post-PD soils (**C**) has still not reached the squaring (74 DAP). Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought.

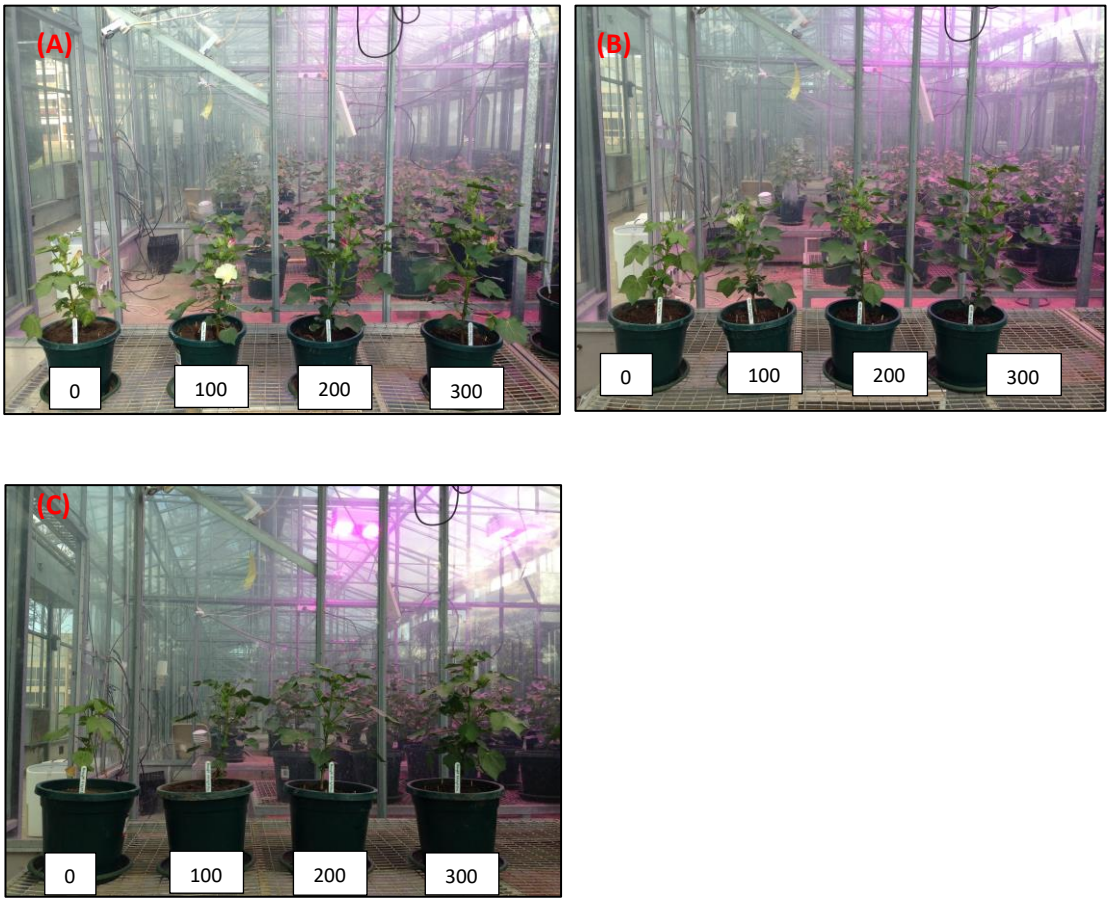

Figure 4.5 Cotton plants grown in glasshouse with different N fertilizer rates including 0, 100, 200 and 300 kg N/ha. Cotton plants grown on (**A**) control and (**B**) Post-WL soils were at the early flowering stage (95 DAP) whereas plants grown on Post-PD soils (**C**) were still at the squaring stage (95 DAP). Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought.

Table 4.5 Plant growth measurements including plant height and number of nodes per plant at different development stages across all soil water treatments and different N-fertilizer rates. Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought. Data are presented as means of four replicates ± SE. For each developmental stage, different letters indicate significant differences between control and Post-WL plants, and control and Post-PD plants at each N level and among N levels.

Stage	Treatment	Plant height				Node per plant			
		0 kg N/ha	100kg N/ha	200 kg N/ha	300 kg N/ha	0 kg N/ha	100 kg N/ha	200 kg N/ha	300 kg N/ha
Early squaring	**Control**	31 ± 0.70d	40 ± 1.19c	48 ± 1.19b	56 ± 2.39a	7 ± 0.40d	9.50 ± 0.28c	11 ± 0.40b	12 ± 0.28a
	Post-WL	20 ± 0.62e	37 ± 1.03c	46 ± 1.25b	52 ± 0.91a	5.70 ± 0.28e	8.70 ± 0.40c	10.76 ± 0.47b	11.80 ± 0.21a
	Post-PD	18 ± 0.71k	27 ± 1.18h	35 ± 1.37g	40 ± 0.81f	5 ± 0.40k	6.10 ± 0.28h	7.40 ± 0.25g	8.70 ± 0.25f
Early flowering	**Control**	40 ± 1.19d	52 ± 0.50c	60 ± 1.08b	67 ± 2.08a	10 ± 0.41d	12 ± 0.26c	13 ± 0.41b	14.54 ± 0.47a
	Post-WL	38 ± 0.85d	50 ± 0.62c	58 ± 0.91b	65 ± 2.29a	9.35 ± 0.62d	11.56 ± 0.40c	12.68 ± 0.47b	14 ± 0.34a
	Post-PD	29 ± 1.04h	38 ± 2.15g	47 ± 0.86f	56.20 ± 2.00e	6 ± 0.24h	8 ± 0.57g	9 ± 0.28f	11 ± 0.25e
Harvest	**Control**	41 ± 0.75d	57 ± 2.49c	68 ± 2.19b	76 ± 2.29a	10 ± 0.25d	14.5 ± 0.24c	15.60 ± 0.45b	17 ± 0.41a
	Post-WL	40 ± 1.22d	55 ± 1.10c	66 ± 1.27b	73 ± 1.31a	10 ± 0.47d	14 ± 0.32c	15 ± 0.25b	16.68 ± 0.40a
	Post-PD	32 ± 1.37h	41 ± 2.10 g	52 ± 1.10f	62 ± 2.40e	8 ± 0.74h	10 ± 0.57g	12.50 ± 0.30f	14.30 ± 0.70e

At harvest, a number of plant measurements were conducted as listed in **Table 4.6**. Seed cotton yield was determined after plants were harvested. Seed cotton yield varied from 13.83 to 57.42; 12.81 to 55.75; and 8.15 to 36.23 g across all N-fertilizer rates for plants grown on control, Post-WL and Post-PD soils, respectively. Total dry biomass of control and plants grown on Post-WL soils varied between 44.85 and 145.02; 39.64 and 136.60 g, respectively, whereas that of plants grown on Post-PD soils was in the range of 20.82 to 110.14 g across all N-fertilizer rates (**Figure 4.6**).

Leaf number varied from 21 to 56, 19 to 54, and 14 to 36 leaves for plants grown on control, Post-WL and Post-PD soils, respectively. Leaf area were in ranges of 1407-4681, 1244-4510, and 579-2292 cm^2 for plants grown on control, Post-WL and Post-PD soils. Boll number and boll size varied from 4 to 15 bolls and 2.5 to 3.4 cm for control plants across all N-fertilizer rates. For Post-WL plants, they varied 4 to 13 bolls and 2.5 to 3.2 cm. Boll number and size of Post-PD plants varied from 2 to 9 bolls and 2.1 to 2.9 cm, respectively. Leaf dry mass varied from 6.95 to 21.02; 6.27 to 20.72; and 3.92 to 14.17 g for plants grown on control, Post-WL and Post-PD soils across all N-fertilizer rates, respectively. Stem dry mass of control, Post-WL and Post-PD plants varied from 12.01 to 33.53, 12.12 to 31.71, and 6.62 to 24.61 g, respectively across all N-fertilizer rates. Root dry mass of control and plants grown on Post-WL soils varied from 6.22 to 12.54 and 5.91 to 11.86 g while that of plants grown on Post-PD soils were from 2.23 to 8.71 g across all treatments.

There were same trends for all these above plant measurements. In particular, all measurements including leaf number, leaf area, boll number, boll size, leaf dry mass, stem dry mass and root dry mass increased with the increase in the rate of N fertilizer addition. With the same rate of N fertilizer, no significant differences in these measurements between control and Post-WL plants were observed while there were statistically significant differences between control and Post-PD plants (**Table 4.6**).

Seed N content of plants grown on control, Post-WL and Post-PD soils varied from 18.71 to 30.14, 17.32 to 31.26, and 16.13 to 27.22 g kg^{-1}, respectively across all N treatments.

Seed C content varied from 517.82 to 528.21, 516.34 to 530.45, and 510.57 to 530.81 g kg^{-1} for plants grown on control, Post-WL and Post-PD soils, respectively. Leaf N and C content of control plants varied from 10.43 to 13.92 g kg^{-1} and 420.61 to 431.21 g kg^{-1}, respectively. Leaf N and C content of Post-WL plants varied from 10.4 to 14.1 g kg^{-1} and 420.83 to 430.20 g kg^{-1}, respectively. Leaf N and C content of Post-PD plants varied from 9.11 to 12.42 g kg^{-1} and 418.16 to 429.22 g kg^{-1}, respectively. Stem N content of control, Post-WL and Post-PD plants varied from 7.11 to 8.74; 7.10 to 8.83, and 6.45 to 8.02 g kg^{-1}, respectively. Stem C content of control, Post-WL and Post-PD plants varied from 441.1 to 454.23; 438.41 to 455.02; and 435.83 to 452.32 g kg^{-1}, respectively. Root N content of control, Post-WL, and Post-PD plants were in the ranges of 5.22 to 5.81, 5.03 to 6.04, and 4.85 to 5.32 g kg^{-1}, respectively. No significant differences in seed, leaf, stem and root N content between control and Post-WL plants were observed at each N addition rate. Seed N of Post-PD plants was significantly lower than that of control plants. However, the content of N in seed, leaf and stem increased with the increased rate of N addition for all soil water treatments. Similarly, no significant differences in seed, leaf, stem, root C content across all soil water treatments and N fertilizer addition rates were observed (**Table 4.6**).

Two-way ANOVA showed significant effects of N-addition on all plant measurements at harvest for Post-WL and Post-PD soils (P<0.001). Prolonged-drought period placed a strong legacy effect on plant measurements whereas waterlogging did not (**Table 4.7**). There was only significant interactive effect of previous waterlogging event and N-addition on seed N content (P=0.044). There were also significant interactive effects of previous pro-longed drought and N-addition on plant measurements at harvest except leaf dry mass and root dry mass and (**Table 4.7**).

Table 4.6 Plant measurements at harvest across all soil water treatments and N fertilizer rates. Data are presented as means of four replicates ± SE of each treatment. Different letters within the same row indicate significant differences between plants grown on control and Post-WL soils, and control and Post-PD soils at each N level and among N levels. Post-WL = Post- waterlogging; Post-PD = Post-prolonged drought. N = Nitrogen, C = Carbon.

Measurements	Control				Post-WL				Post-PD			
	0 kg N/ha	100 kg N/ha	200 kg N/ha	300 kg N/ha	0 kg N/ha	100 kg N/ha	200 kg N/ha	300 kg N/ha	0 kg N/ha	100 kg N/ha	200 kg N/ha	300 kg N/ha
Leaf number	21 ± 1.82d	40 ± 3.34c	49 ± 0.7b	56 ± 1.95a	19.5 ± 1.04d	38.75 ± 2.56c	48.25 ± 3.4b	54.8 ± 3.02a	14.5 ± 0.86h	25 ± 2.48g	33 ± 2.12f	36 ± 2.67e
Leaf area (cm^2)	1267 ± 120.52d	2901.34 ± 416.81c	4142 ± 64.11b	4578 ± 124.75a	1244 ± 120.06d	2891.75 ± 21.78c	4149 ± 146.41b	4510.25 ± 63.29a	579.5 ± 76.2h	1155.5 ± 117.38g	1711.5 ± 264.69f	2292 ± 87.09e
Boll number	4 ± 0.25d	7.6 ± 0.4c	13 ± 0.47b	15.2 ± 1.08a	3.7 ± 0.25d	7 ± 0.7c	12.8 ± 0.25b	14.5 ± 0.75a	2 ± 0.25h	5 ± 0.25g	7 ± 0.75f	9 ± 0.71e
Boll size (cm)	2.5 ± 0.08d	2.95 ± 0.06c	3.32 ± 0.04b	3.48 ± 0.05a	2.53 ± 0.05d	2.87 ± 0.09c	3.25 ± 0.02b	3.41 ± 0.085a	2.01 ± 0.04h	2.39 ± 0.05g	2.67 ± 0.03f	2.94 ± 0.04e
Leaf dry mass (g)	6.95 ± 0.58d	12.05 ± 1.03c	17.3 ± 0.86b	21 ± 0.62a	6.27 ± 0.68d	11 ± 1.16c	16.37 ± 0.49b	20.07 ± 0.52a	3.92 ± 0.81h	7.97 ± 0.9g	11.9 ± 0.49f	14.17 ± 0.32e
Stem dry mass (g)	12.05 ± 0.47d	24.42 ± 1.13c	29.65 ± 1.52b	33.55 ± 1.01a	12.05 ± 2.9d	24.4 ± 0.4c	27.1 ± 0.95b	31.77 ± 1.18a	6.6 ± 0.69h	16.47 ± 0.23g	19.13 ± 1.25f	24.6 ± 0.51e
Root dry mass (g)	6.2 ± 0.44d	8.51 ± 0.47c	10.45 ± 0.4b	12.58 ± 0.41a	5.98 ± 0.33d	7.8 ± 0.61c	9.94 ± 0.27b	11.83 ± 0.61a	2.25 ± 0.26h	4.78 ± 0.64g	7.93 ± 0.11f	8.74 ± 0.31e
Seed N (g kg^{-1})	18.76 ± 1.02d	23.51 ± 1.12c	27.97 ± 0.26b	30.12 ± 2.69a	17.31 ± 0.71d	22.34 ± 1.89c	27.25 ± 1.04b	31.25 ± 0.41a	16.09 ± 0.78h	20.07 ± 0.7g	23 ± 0.96f	27.19 ± 0.67e
Seed C (g kg^{-1})	517.78 ± 5.07c	529.76 ± 3.38ab	528.23 ± 3.14ab	528.23 ± 3.14ab	516.29 ± 3.06c	526.5 ± 2.1b	529 ± 1.74ab	530.36 ± 1.33a	510.54 ± 4.66c	521.83 ± 1.08a	525.11 ± 2.87a	530.82 ± 2.35a
Leaf N (g kg^{-1})	10.36 ± 0.63c	12.07 ± 1.04ab	13.45 ± 0.63a	13.92 ± 1.12a	10.42 ± 0.31c	12.56 ± 0.74a	13.87 ± 1.06a	14.03 ± 0.68a	9.04 ± 0.56d	10.09 ± 0.24c	11.21 ± 0.67b	12.42 ± 0.32a
Stem N (g kg^{-1})	7.14 ± 0.45c	8.03 ± 0.12a	8.37 ± 0.45a	8.72 ± 0.63a	7.13 ± 0.36c	8.10 ± 0.23b	8.29 ± 0.41ab	8.81 ± 0.34a	6.43 ± 0.15d	7.96 ± 0.32a	7.68 ± 0.26a	8.03 ± 0.41a
Root N (g kg^{-1})	5.16 ± 0.25b	5.79 ± 0.12a	5.63 ± 0.34a	5.81 ± 0.51a	5.02 ± 0.32b	5.62 ± 0.17a	5.85 ± 0.23a	6.01 ± 0.38a	4.78 ± 0.56ab	5.01 ± 0.31ab	5.31 ± 0.22ab	5.29 ± 0.35ab

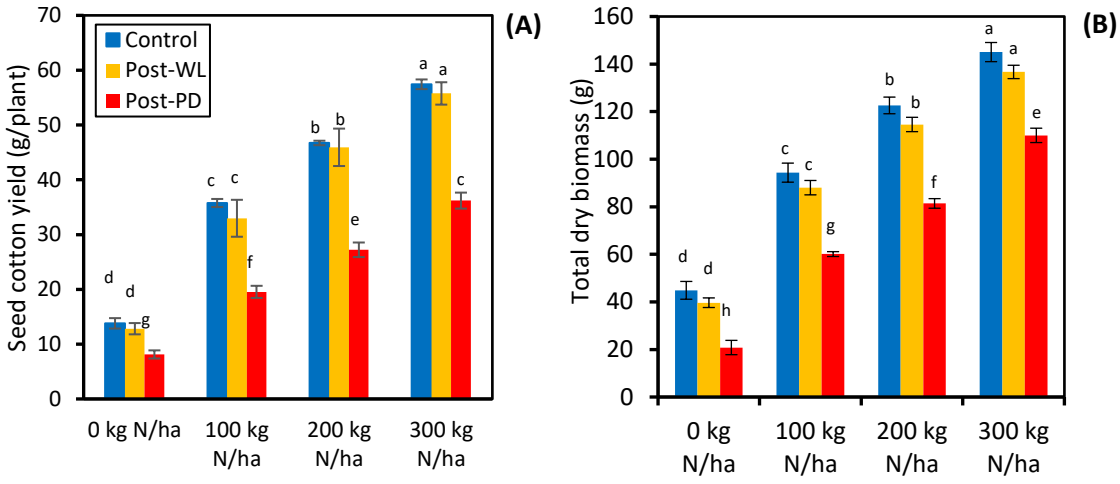

Figure 4.6 Seed cotton yield **(A)** and total biomass **(B)** of cotton plants at harvest across all treatments. Data are presented as means of four replicates ± SE of each treatment. Different letters indicate significant differences between control and Post-WL plants, and control and Post-PD plants at each N level and among N levels. Post-WL = Post-waterlogging. Post-PD = Post-prolonged drought.

Table 4.7 Two-way ANOVA for the effects of N-addition, soil water treatment legacy and their interaction on plant growth measurements and productivity. Significant differences at $P<0.05$ are in bold. Post-WL= Post-waterlogging, Post-PD = Post-prolonged drought, C = Carbon, N = Nitrogen.

Factor	N-addition		Soil water treatment legacy		N-addition x soil water treatment legacy	
	Post-WL	Post-PD	Post-WL	Post-PD	Post-WL	Post-PD
Leaf number	**<0.001**	**<0.001**	0.192	**<0.001**	0.6	**0.015**
Leaf area	**<0.001**	**<0.001**	0.192	**<0.001**	0.998	**0.001**
Boll number	**<0.001**	**<0.001**	0.234	**<0.001**	0.615	**<0.001**
Boll size	**<0.001**	**0.001**	0.451	**<0.001**	0.55	**0.012**
Leaf dry mass	**<0.001**	**<0.001**	0.177	**<0.001**	0.924	0.086
Stem dry mass	**<0.001**	**<0.001**	0.105	**<0.001**	0.48	**<0.001**
Root dry mass	**<0.001**	**<0.001**	0.089	**<0.001**	0.935	0.335
Seed cotton yield	**<0.001**	**<0.001**	0.272	**<0.001**	0.956	**<0.001**
Seed C	**0.001**	**0.019**	0.815	0.255	0.836	0.696
Seed N	**<0.001**	**<0.001**	0.914	**<0.001**	0.839	0.826
Total dry mass	**<0.001**	**<0.001**	0.059	**<0.001**	0.682	**<0.001**
Plant node	**<0.001**	**<0.001**	0.603	**<0.001**	0.17	0.504
Plant height	**<0.001**	**<0.001**	0.42	**<0.001**	0.993	0.711
Leaf N	0.001	<0.001	0.621	0.002	0.993	0.871
Stem N	0.004	0.002	0.911	0.065	0.991	0.765
Root N	0.076	0.357	0.911	0.056	0.884	0.953

4.3.2.2 Soil physicochemical properties

Soil moisture was approximately 25.33% in average for all pots and consistent across all sampling time points. Soil pH varied from 6.71 to 7.93, 6.75 to 8.00, and 6.91 to 8.01 for control, Post-WL, and Post-PD soils, respectively across all treatments and developmental stage. Soil NH_4^+ concentration of control, Post-WL, and Post-PD soils varied from 0.77 to 10.46, 0.64 to 10.05, and 0.16 to 6.81 mg/kg, respectively across all treatments and developmental stages (**Table 4.8**). No significant differences in soil NH_4^+ were observed between control and Post-WL soils at each developmental stage while NH_4^+ concentration of Post-PD soils was significantly lower than that of control soils. Soil NH_4^+ significantly increased when the rate of N-fertilizer addition increased (**Table 4.8**).

Soil NO_3^- concentration of control, Post-WL and Post-PD soils varied from 4.32 to 195.61; 4.17 to 198.77; and 1.84 to 125.83 mg/kg dry soil, respectively across all treatments and developmental stages. At each developmental stage, NO_3^- concentration significantly increased with the increase in N-fertilizer addition rate. At the same rate of N-fertilizer addition, there were statistically significant differences in NO_3^- concentration between control and Post-PD soils. In contrast, no significant differences were observed for control and Post-WL soils at the rates of 100, 200 and 300 kg N/ha; however, NO_3^- concentration of control and Post-WL soils were significantly different when no N-fertilizer was added (**Table 4.8**).

Two-way repeated measures ANOVA showed overall significant effects of N-addition on soil physicochemical properties. Previous waterlogging events did not significantly affect soil physicochemical properties (**Table 4.9**). Prolonged-drought prior to sowing established significant effects on all soil physicochemical properties (P<0.001). Significant interactive effects of previous prolonged-drought and N-addition were observed for NH_4^+, NO_3^- (P<0.001). The growth stages showed significant effects on soil physicochemical properties (P<0.05). No interactive effects of N-addition and growth stage on pH of Post-WL soils were observed (P=0.404). There were interactive effects of growth stage and legacy effect of prolonged-drought on soil NH_4^+ and NO_3^- contents;

growth stage and legacy effect of waterlogging on soil NO_3^- content. The interactive effect of N-addition x prolonged-drought x growth stage was only found for soil NO_3^- content (P<0.001) (**Table 4.9**).

Table 4.8 Soil physicochemical properties across all soil water treatments and N-fertilizer rates. Data were recorded for different developmental stages. Data are presented as means of four replicates ± SE of each treatment. Different letters within the same row indicate significant differences between control and Post-WL soils, and control and Post-PD soils at each N level and among N levels. Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought, NH_4^+ = Ammonium, NO_3^- = Nitrate, C = Carbon, C = Nitrogen.

Stages	Control				Post-WL				Post-PD			
	0 kg N/ha	100 kg N/ha	200 kg N/ha	300 kg N/ha	0 kg N/ha	100 kg N/ha	200 kg N/ha	300 kg N/ha	0 kg N/ha	100 kg N/ha	200 kg N/ha	300 kg N/ha
Early squaring												
Soil moisture	24.94 ± 1.79a	24.35 ± 0.73a	25.12 ± 1.66a	25.06 ± 0.61a	25.53 ± 1.80a	23.86 ± 1.05a	24.10 ± 1.03a	24.24 ± 1.99a	23.74 ± 1.23a	25.37 ± 0.78a	25.14 ± 1.07a	24.44 ± 0.67a
pH	7.90 ± 0.06a	7.42 ± 0.06b	7.38 ± 0.08b	6.71 ± 0.09c	8.00 ± 0.06a	7.61 ± 0.13b	7.12 ± 0.07c	6.75 ± 0.04c	8.00 ± 0.08a	7.75 ± 0.15b	7.24 ± 0.10b	6.91 ± 0.10c
NH_4^+	2.84 ± 0.14d	5.75 ± 0.42c	8.62 ± 0.54b	10.46 ± 0.32a	2.31 ± 0.53d	4.86 ± 0.45c	8.33 ± 0.39b	10.05 ± 0.33a	1.20 ± 0.17h	2.73 ± 0.51g	4.96 ± 0.18f	6.80 ± 0.34e
NO_3^-	12.21 ± 0.42d	87.33 ± 1.10c	176.68 ± 1.84b	195.61 ± 2.76a	7.47 ± 1.64e	84.15 ± 1.99c	169.34 ± 2.21b	198.77 ± 3.76a	7.04 ± 0.86h	65.95 ± 2.26g	101.29 ± 1.77f	125.83 ± 2.96e
Total N	0.61 ± 0.01d	0.89 ± 0.02c	1.07 ± 0.02b	1.21 ± 0.09a	0.57 ± 0.07d	0.81 ± 0.05c	1.04 ± 0.04b	1.18 ± 0.03a	0.37 ± 0.02h	0.66 ± 0.01g	0.80 ± 0.02f	0.95 ± 0.06e
Total C	8.46 ± 0.21a	8.51 ± 0.15a	8.54 ± 0.11a	9.01 ± 0.44a	8.16 ± 0.36a	8.47 ± 0.21a	8.58 ± 0.13a	8.87 ± 0.31a	7.11 ± 0.14b	7.23 ± 0.09b	7.45 ± 0.20b	7.81 ± 0.45b
Early flowering												
Soil moisture	26.15 ± 2.31a	25.70 ± 0.65a	26.10 ± 0.81a	25.79 ± 1.46a	25.58 ± 1.34a	26.18 ± 2.01a	24.67 ± 2.33a	26.31 ± 2.43a	25.98 ± 1.28a	26.03 ± 2.40a	25.72 ± 2.20a	25.47 ± 1.69a
pH	7.93 ± 0.16a	7.52 ± 0.10b	7.28 ± 0.07c	7.14 ± 0.03d	7.85 ± 0.26a	7.73 ± 0.07b	7.31 ± 0.07c	7.12 ± 0.09d	7.72 ± 0.16a	7.67 ± 0.04b	7.41 ± 0.05c	7.45 ± 0.20c
NH_4^+	1.60 ± 0.17d	3.18 ± 0.32c	4.31 ± 0.24b	6.87 ± 0.26a	1.35 ± 0.26d	3.12 ± 0.41c	3.98 ± 0.13b	6.81 ± 0.56a	0.65 ± 0.15h	1.12 ± 0.12g	2.48 ± 0.39f	4.80 ± 0.44e
NO_3^-	7.27 ± 0.34d	68.51 ± 3.70c	129.96 ± 1.51b	161.54 ± 1.80a	6.03 ± 0.16d	65.52 ± 1.23c	127.99 ± 2.45b	157.56 ± 1.72a	4.07 ± 0.25h	46.42 ± 2.17g	80.72 ± 2.33f	106.89 ± 1.19e

Table 4.8 (Cont.)

Stages	Control				Post-WL				Post-PD			
	0 kg N/ha	100 kg N/ha	200 kg N/ha	300 kg N/ha	0 kg N/ha	100 kg N/ha	200 kg N/ha	300 kg N/ha	0 kg N/ha	100 kg N/ha	200 kg N/ha	300 kg N/ha
Early flowering												
Total N	0.52 ± 0.01d	0.79 ± 0.04c	0.96 ± 0.02b	1.14 ± 0.04a	0.48 ± 0.03d	0.72 ± 0.03c	0.91 ± 0.03b	1.12 ± 0.02a	0.31 ± 0.01h	0.52 ± 0.01g	0.67 ± 0.02f	0.86 ± 0.03e
Total C	8.21 ± 0.11b	8.42 ± 0.23ab	8.77 ± 0.38ab	8.82 ± 0.17a	8.01 ± 0.24b	8.39 ± 0.17ab	8.53 ± 0.32ab	8.79 ± 0.41a	7.02 ± 0.18d	7.21 ± 0.23cd	7.38 ± 0.31cd	7.76 ± 0.42c
Harvest												
Soil moisture	24.69 ± 2.03a	26.47 ± 0.72a	25.77 ± 1.42a	26.70 ± 1.49a	25.55 ± 2.16a	25.80 ± 2.79a	25.46 ± 0.78a	25.24 ± 1.15a	24.63 ± 2.89a	24.87 ± 1.26a	26.73 ± 0.88a	26.59 ± 1.02a
pH	7.90± 0.17a	7.78 ± 0.10a	7.89 ± 0.12a	7.69 ± 0.45a	8 ± 0.09a	7.90 ± 0.15a	7.93 ± 0.27a	7.73 ± 0.33a	8.01 ± 0.15a	7.93 ± 0.06a	7.88 ± 0.06a	7.89 ± 0.11a
NH_4^+	0.77 ± 0.07d	0.81 ± 0.07c	1.33 ± 0.09b	2.19 ± 0.08a	0.64 ± 0.09d	0.76 ± 0.04c	1.21 ± 0.06b	2.01 ± 0.07a	0.16 ± 0.04h	0.32 ± 0.04g	0.62 ± 0.02f	0.97 ± 0.02e
NO_3^-	4.32 ± 0.21d	9.17 ± 0.60c	14.11 ± 0.45b	18.89 ± 0.40a	4.17 ± 0.62d	9.88 ± 0.23c	13.67 ± 0.32b	18.65 ± 0.23a	1.84 ± 0.67h	5.28 ± 0.27g	7.89 ± 0.68f	10.78 ± 0.14e
Total N	0.46 ± 0.04d	0.68 ± 0.02c	0.86 ± 0.02b	0.92 ± 0.01a	0.42 ± 0.01d	0.63 ± 0.03c	0.82 ± 0.04b	0.92 ± 0.01a	0.26 ± 0.01h	0.45 ± 0.02g	0.55 ± 0.01f	0.76 ± 0.01e
Total C	7.82 ± 0.15b	8.19 ± 0.17a	8.27 ± 0.23a	8.56 ± 0.19a	7.97 ± 0.46ab	8.20 ± 0.21a	8.21 ± 0.17a	8.60 ± 0.24a	6.67 ± 0.23d	7.01 ± 0.36cd	7.16 ± 0.15cd	7.63 ± 0.31c

Table 4.9 Two-way repeated measures ANOVA for the effect of N-addition (**N**), soil water treatment legacy (**W**), cotton growth stage (**T**) and their interaction on soil properties. Significant effects are in bold (P<0.05). Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought. NH_4^+ = Ammonium, NO_3^- = Nitrate, Total C = Total carbon, Total N = Total nitrogen.

Factor	pH		NH_4^+		NO_3^-		Total C		Total N	
	Post-WL	Post-PD	Post-WL	Post-PD	Post-WL	Post-PD	Post-WL	Post-PD	Post-WL	Post-PD
N	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.186	0.094	**<0.001**	**<0.001**
W	0.666	**0.001**	0.051	**<0.001**	0.074	**<0.001**	0.230	**0.017**	0.088	**<0.001**
N x W	0.072	0.596	0.865	**0.005**	0.799	**<0.001**	0.620	0.832	0.584	0.231
T	0.285	**0.003**	**0.010**	**<0.001**	**<0.001**	**<0.001**	**0.041**	**0.023**	**<0.001**	**<0.001**
N x T	0.404	**0.002**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.650	0.920	**0.016**	0.057
W x T	0.758	0.317	0.410	**<0.001**	**0.001**	**<0.001**	0.132	0.754	0.862	0.492
N x W x T	0.223	0.086	0.975	0.188	0.971	**<0.001**	0.923	0.160	0.999	0.382

4.3.2.3 Soil processes

4.3.2.3.1 Potential nitrification rate

Potential nitrification rate (PNR) was measured for samples collected at the early squaring, early flowering stages, and harvest. At the early squaring, PNR varied from 0.44 to 1.33 mg NO_2-N/kg dry soil/h for control soils across all N-fertilizer rates. PNR of samples collected from Post-WL soils changed in the range of 0.41 to 1.29 mg NO_2-N/kg dry soil/h whereas it was 0.23 to 0.91 mg NO_2-N/kg dry soil/h for Post-PD soils. At the early flowering stage, in general, PNR of all soils were higher than that of samples collected at the early squaring. PNR of control soils varied from 0.67 to 1.57 mg NO_2-N/kg dry soil/h across all N-fertilizer rates whereas that of Post-PD soils varied from 0.31 to 1.12 mg NO_2-N/kg dry soil/h. At harvest, the PNR varied from 0.44 to 1.18, 0.45 to 1.11 and 0.26 to 0.88 mg NO_2-N/kg dry soil/h for control, Post-WL, and Post-PD soils (**Figure 4.7**).

There are the same trends at each developmental stage. In particular, PNR of Post-PD soils were significantly lower than that of control at each N-fertilizer rate. In contrast, no significant differences were observed for samples collected from control and Post-WL soils. PNR increased with the increase in the rate of N-fertilizer addition for all soil treatments (**Figure 4.7**).

Two-way repeated measures ANOVA showed overall significant effects of N-addition and growth stage, and N-addition x growth stage on PNR of Post-WL and Post-PD soils (P< 0.001). Prolonged-drought placed a legacy effect on PNR and the interaction of prolonged-drought x growth stage significantly affected PNR (P<0.001) (**Table 4.10**).

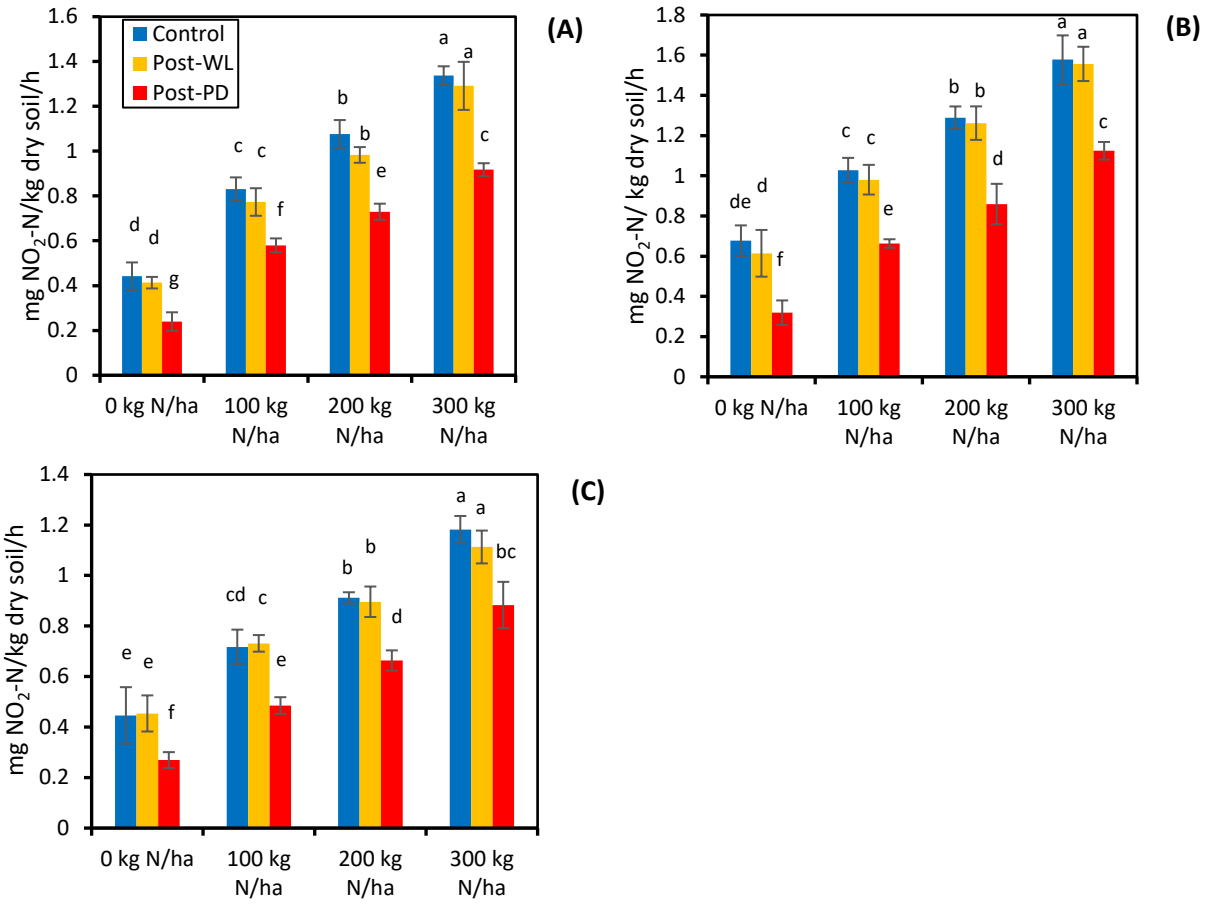

Figure 4.7 Potential nitrification rate (PNR) of different previously water-treated soils which were applied different N-fertilizer rates. Data present PNR at (**A**) the early squaring stage, (**B**) the early flowering stage, and (**C**) final harvest. Values represent mean ± SE (n=4) of each soil water treatment at each N level. Different letters indicate significant differences between control and Post-WL soils, and control and Post-PD soils at each N level and among N levels. Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought.

4.3.2.3.2 Net nitrification and net N mineralization

Net nitrification rate (NNR) varied from 0.16 to 0.54 mg NO_3^--N/kg dry soil/day for control soils. NNR varied from 0.15 to 0.48 and 0.10 to 0.34 mg NO_3^--N/kg dry soil/day for Post-WL and Post-PD soils, respectively. NNR significantly increased when the amount of N-fertilizer addition increased for all soil water treatments. There were significant differences in NNR between control and Post-PD soils when the same amount of N-fertilizer was applied. In contrast, no significant differences in NNR were observed between control and Post-WL soils at each N-fertilizer addition rate (**Figure 4.8A**).

Net N mineralization rate (NMR) varied from 0.14 to 0.45, 0.14 to 0.48, and 0.08 to 0.31 mg N/kg dry soil/day for control, Post-WL and Post-PD soils, respectively. No significant differences in NMR between control and Post-WL soils at each N levels whereas NMR of Post-PD soils was significantly lower than that of control. NMR increased with the increase in N fertilizer rates for all soil water treatments (**Figure 4.8B**).

Two-way repeated measures ANOVA indicated that N-addition, growth stage, and N-addition x growth stage had significant effects on NNR and NMR ($P<0.05$). There were legacy effects of prolonged-drought on NNR and NMR ($P<0.001$, $P=0.01$, respectively). Similarly, N-addition x prolonged-drought significantly affected NMR ($P=0.03$) (**Table 4.10**).

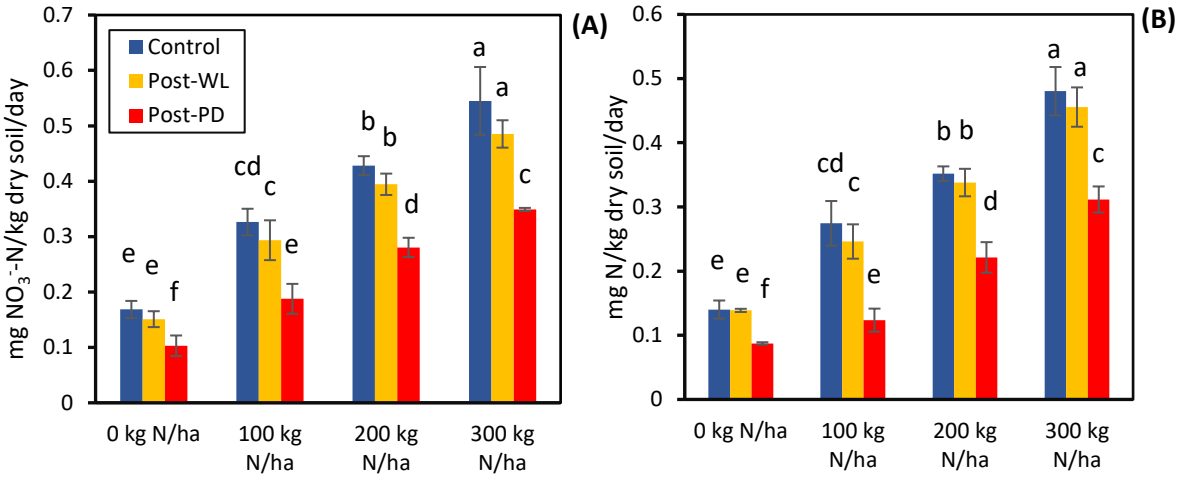

Figure 4.8 Net nitrification (**A**) and net N mineralization (**B**) rates across all soil water treatments and N fertilizer rates at harvest. Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought. Values represent mean ± SE (n=4) of each treatment. Different letters indicate significant differences between control and Post-WL soils; and control and Post-PD soils at each N levels and among N levels.

4.3.2.3.3 Potential denitrification rate

Potential denitrification rates (PDR) were measured for samples collected at the early squaring and flowering stages and harvest. At the early squaring stage, PDR of control samples varied from 0.05 to 0.38 µg N_2O-N/g dry soil/h across all N-fertilizer rates whereas those of Post-WL and Post-PD soils varied from 0.08 to 0.49 and 0.02 to 0.29 µg N_2O-N/g dry soil/h, respectively. At the early flowering stage, PDR of control soils varied from 0.12 to 0.42 µg N_2O-N/g dry soil/h. PDR of Post-WL and Post-PD soils varied from 0.04 to 0.62 and 0.12 to 0.32 µg N_2O-N/g dry soil/h, respectively. At harvest, PDR of control soils varied from 0.07 to 0.31 µg N_2O-N/g dry soil/h whereas those of Post-WL and Post-PD soils varied from 0.12 to 0.39 and 0.05 to 0.2 µg N_2O-N/g dry soil/h (**Figure 4.9**).

At each developmental stage, PDR of control and Post-WL samples were significantly different when the same amount of N-fertilizer was applied. The same trends were observed when comparing control and Post-PD soils. Additionally, PDR had an upward trend when the rate of N-fertilizer increased (**Figure 4.9**).

Two-way repeated measures ANOVA showed significant effects of N-addition, prolonged-drought, and waterlogging (soil water treatments), N-addition x soil water treatment, growth stage, N-addition x growth stage on PDR. Additionally, waterlogging x growth stage, and N-addition x waterlogging x growth stage had significant effects on PDR (**Table 4.10**).

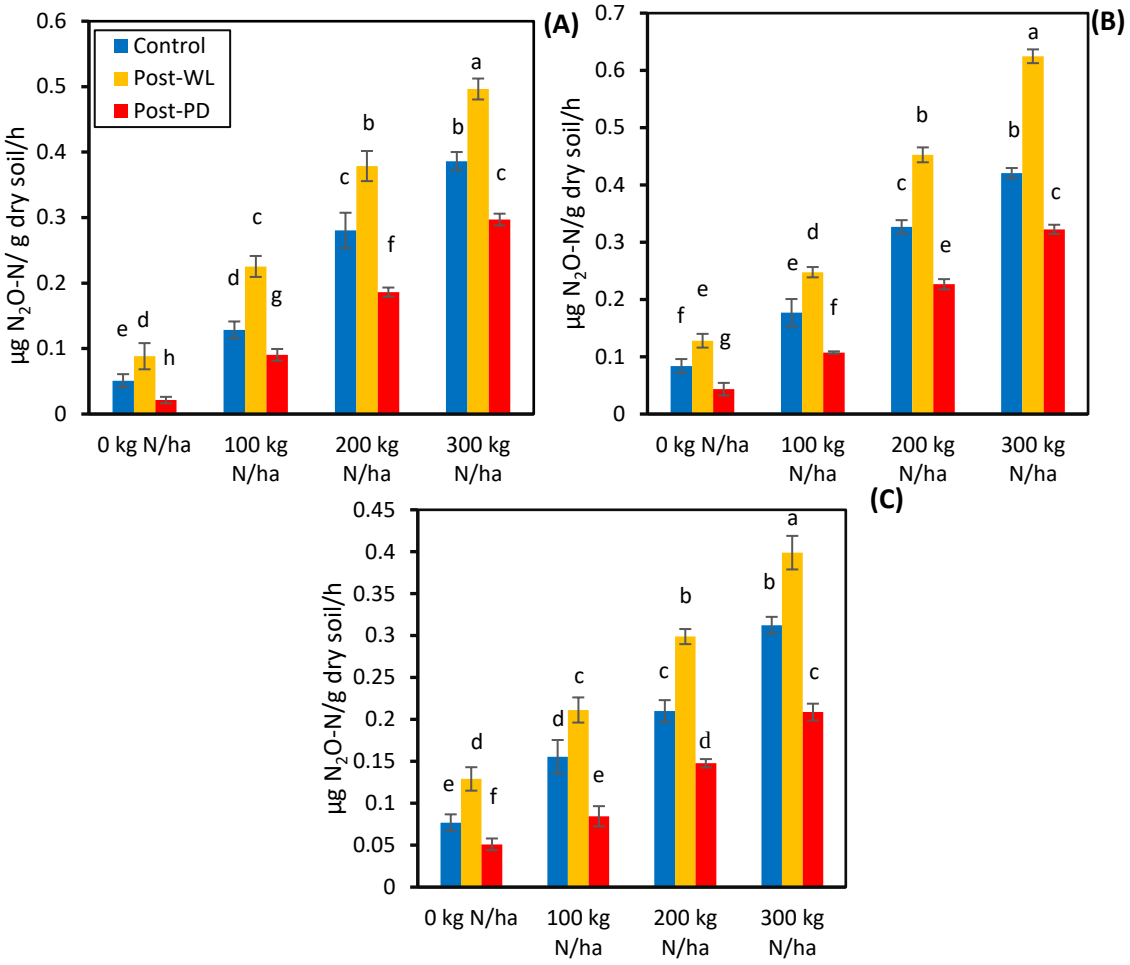

Figure 4.9 Potential denitrification rate (PDR) of samples collected at (**A**) the early squaring stage, (**B**) the early flowering stage, and (**C**) harvest across all soil water treatments and N fertilizer rates. Values represent mean ± SE (n=4) of each soil water treatment at each N level. Different letters indicate statistically significant differences between control and Post-WL soils; and control and Post-PD soils at each N level and among N levels. Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought.

Table 4.10 Two-way repeated measures ANOVA for the effect of N-addition (**N**), soil water treatment legacy (**W**), cotton growth stage (**T**) and their interaction on soil processes including potential nitrification rate (PNR), potential denitrification rate (PDR), net nitrification and N mineralization rate (NNR and NMR), and microbial respiration rate. Significant effects are in bold (P<0.05). Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought.

Factor	PNR		PDR		NMR		NNR	
	Post-WL	Post-PD	Post-WL	Post-PD	Post-WL	Post-PD	Post-WL	Post-PD
N	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.030**	**0.002**	**<0.001**	**<0.001**
W	0.167	**<0.001**	**<0.001**	**<0.001**	0.670	**0.010**	0.170	**<0.001**
N x W	0.983	0.259	**0.002**	**0.007**	0.450	**0.030**	0.910	0.140
T	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.008**	**0.034**	**<0.001**	**<0.001**
N x T	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.005**	**0.012**	**<0.001**	**<0.001**
W x T	0.917	**0.001**	**0.004**	0.704	0.780	0.090	0.170	0.352
N x W x T	1.000	0.707	**0.011**	0.056	0.480	0.210	0.910	0.140

4.3.2.4 Soil microbial communities

4.3.2.4.1 The AOB and AOA *amoA* gene abundance

The AOB and AOA *amoA* gene abundances were determined for samples collected at the early squaring, early flowering stages, and harvest.

At the early squaring stage, the AOB abundance of control soils ranged from 5.14 x 10^6 to 1.82 x 10^7 copies g^{-1} soil which did not significantly differ from that of Post-WL soils. The AOB abundance of Post-WL soils varied from 4.42 x 10^6 to 1.77 x 10^7 copies g^{-1} soil. For Post-PD soils, the AOB abundance ranged from 1.77 x 10^6 to 1.09 x 10^7 copies g^{-1} soil and significantly lower than that of control soils (**Figure 4.10A**). At the early flowering stage, the AOB *amoA* gene abundance varied from 7.17 x 10^6 to 2.09 x 10^7 copies g^{-1} soil for control samples. In terms of Post-WL and Post-PD soils, the AOB abundances ranged from 6.16 x 10^6 to 2.01 x 10^7, and 2.15 x 10^6 to 1.58 x 10^7 copies g^{-1} soil (**Figure 4.10B**). At harvest, the AOB abundance of control and Post-WL soils varied from 3.86 x 10^6 to 1.57 x 10^7 copies g^{-1} soil and 3.86 x 10^6 to 1.44 x 10^7, respectively whereas that of Post-PD changed between 1.47 x 10^6 and 9.57 x 10^7 copies g^{-1} soil (**Figure 4.10C**).

At each developmental stage, the same trends were observed for the AOB abundance. In particular, the AOB abundance significantly increased when the amount of N addition increased. At the same rate of N-addition, there were significant differences in the AOB abundance between control and Post-PD soils. In contrast, no significant difference was observed between control and Post-WL soils (**Figure 4.10**).

The AOA *amoA* gene abundance of control, Post-WL and Post-PD soils varied from 1.95 x 10^8 to 2.38 x 10^8, 1.87 x 10^8 to 2.14 x 10^8, and 1.76 x 10^8 to 2.29 x 10^8 copies g^{-1} soil, respectively across all treatments and developmental stages. In contrast to AOB abundance, no significant differences in AOA abundance were observed between soil water treatments and N- addition treatments at each developmental stage (**Figure 4.11**).

Two-way repeated measures ANOVA showed overall significant effects of N-addition and growth stage on the AOB abundance of Post-WL and Post-PD soils (P<0.001). Prolonged-drought established a legacy effect on the AOB abundance (P<0.001). The interaction of N addition x prolonged-drought had a significant effect on the AOB abundance (P<0.001) (**Table 4.11**). In contrast to the AOB abundance, waterlogging and prolonged-drought did not establish legacy effects on the AOA abundance. N-addition also did not significantly affect the AOA abundance while growth stage had only marginally significant effects on the AOA abundance (P=0.055 for Post-WL, P=0.075 for Post-PD) (**Table 4.11).**

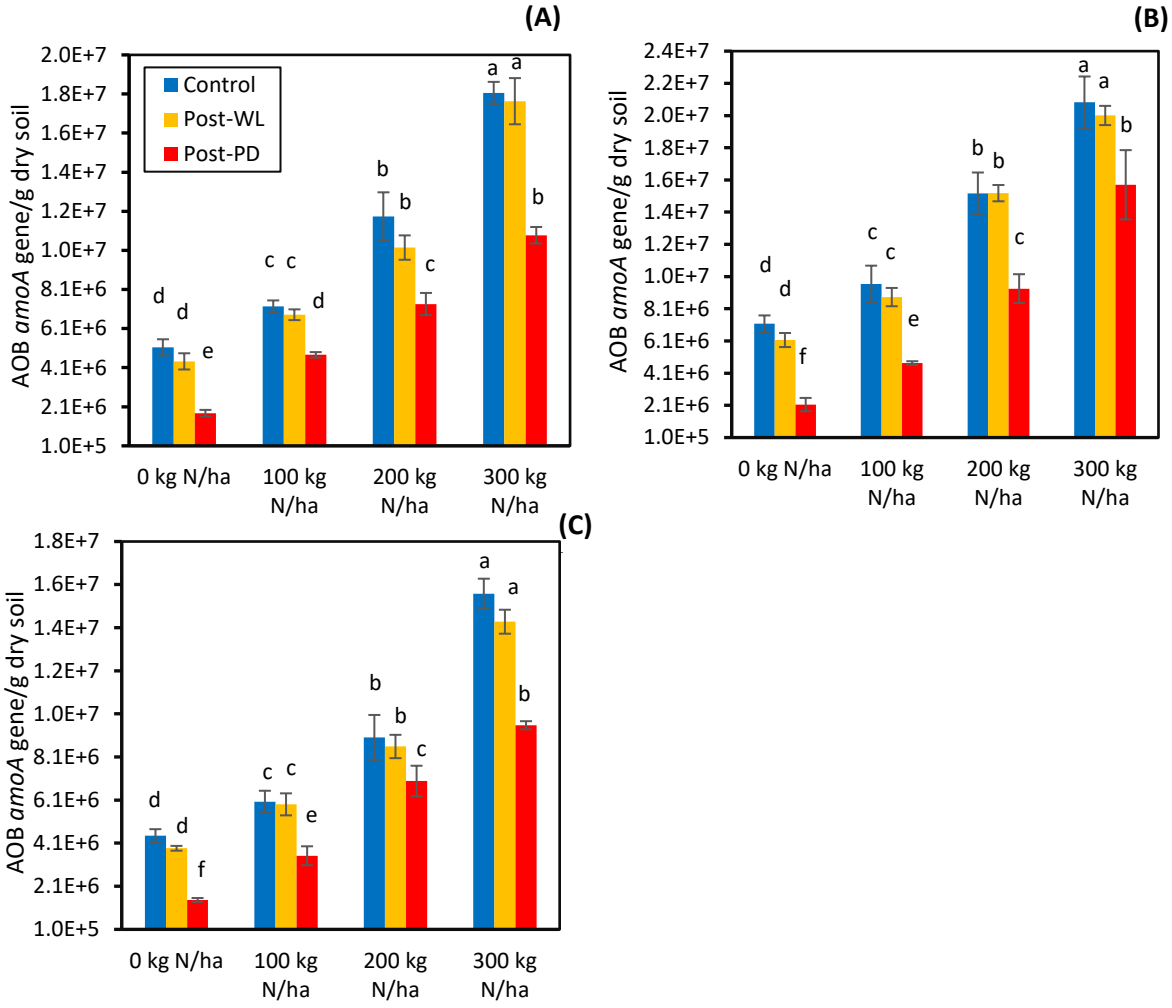

Figure 4.10 The AOB *amoA* gene abundance across all soil treatments with different N fertilizer rates at (**A**) the early squaring, (**B**) the early flowering, and (**C**) harvest. Values represent mean ± SE (n=4) of each soil water treatment at each N level. Different letters indicate statistically significant differences between control and Post-WL soils; and control and Post-PD soils at each N level and among N levels. AOB = Ammonia-oxidizing bacteria. Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought.

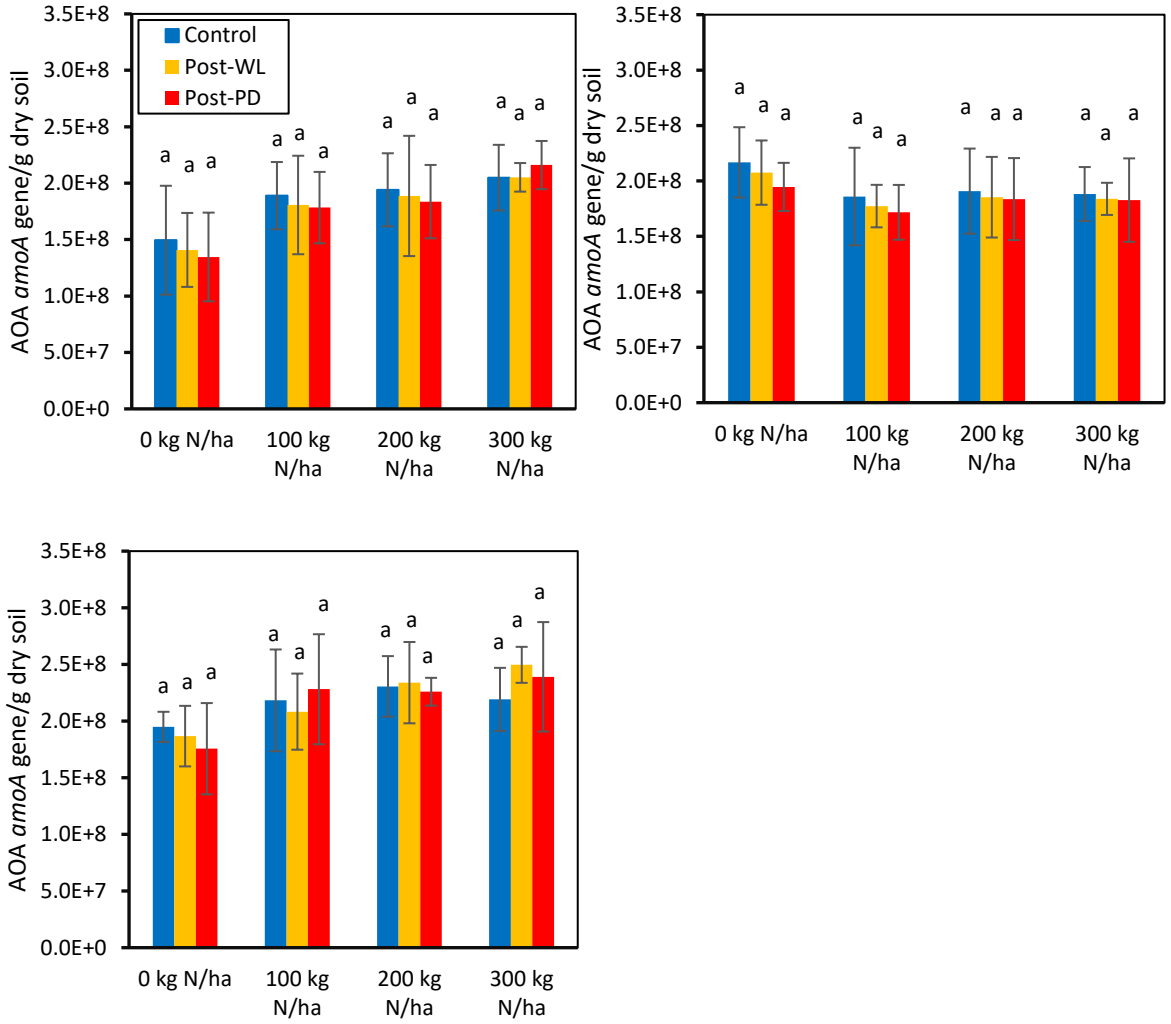

Figure 4.11 The AOA *amoA* gene abundance across all soil treatments with different N fertilizer rates at (**A**) the early squaring, (**B**) the early flowering, and (**C**) harvest. Values represent mean ± SE (n=4) of each soil water treatment at each N level. Different letters indicate statistically significant differences between control and Post-WL soils; and control and Post-PD soils at each N level and among N levels. AOA = Ammonia-oxidizing archaea, Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought.

4.3.2.4.3 The abundance of N_2O-reducing bacteria (*nosZ* gene)

At the early squaring, the *nosZ* gene abundance varied from 1.86 x 10^7 to 9.57 x 10^7 copies g^{-1} soil for samples collected from control pots. Regarding Post-WL and Post-PD soils, the *nosZ* gene abundance varied from 5.87 x 10^7 to 1.22 x 10^8, and from 3.95 x 10^6 to 6.52 x 10^7 copies g^{-1} soil. At the early flowering stage, the *nosZ* gene abundance of control and Post-WL soils ranged from 2.26 x 10^7 to 1.07 x 10^8 and 6.17 x 10^7 -1.43 x 10^8 copies g^{-1} soil. For Post-PD soils, the *nosZ* gene abundance varied from 4.15 x 10^6 to 7.52 x 10^7 copies g^{-1} soil. At harvest, the *nosZ* gene abundance of control soil varied from 1.16 x 10^6 to 9.07 x 10^7 copies g^{-1} soil whereas that of Post-WL and Post-PD varied from 5.12 x 10^7 to 1.02 x 10^8 copies g^{-1} soil and from 3.15 x 10^6 to 5.92 x 10^7 copies g^{-1} soil. At each developmental stage, significant differences in the *nosZ* gene abundance were observed between soil water treatments when the same N fertilizer was applied. Additionally, the *nosZ* gene abundance increased with the increase in N-fertilizer addition for all soil water treatments (**Figure 4.12**).

Two-way repeated measurements ANOVA showed significant effects of N-addition, soil water treatment (waterlogging and prolonged-drought), growth stage, and the interaction of N-addition x soil water treatment on the *nosZ* gene abundance. It means that both waterlogging and prolonged-drought established legacy effects on the *nosZ* gene abundance (**Table 4.11**).

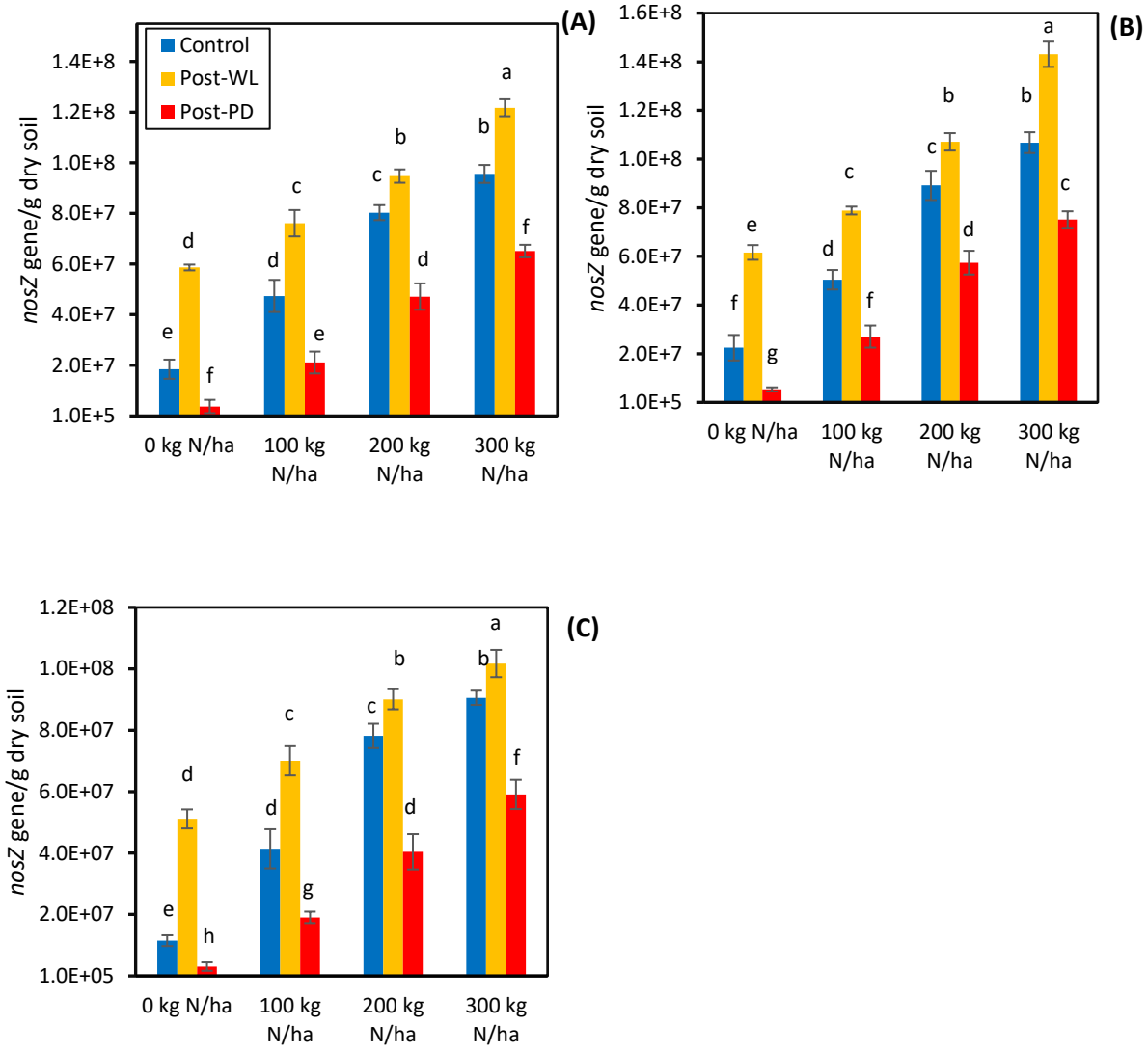

Figure 4.12 The abundance of N_2O-reducing bacteria (*nosZ* gene) across all soil treatments with different N fertilizer rates at (**A**) the early squaring, (**B**) the early flowering, and (**C**) harvest. Values represent mean ± SE (n=4) of each soil water treatment at each N level. Different letters indicate statistically significant differences between control and Post-WL soils; and control and Post-PD soils at each N level and among N levels. Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought, N_2O = Nitrous oxide.

Table 4.11 Two-way repeated measures ANOVA for the effect of N-addition (**N**), soil water treatment legacy (**W**), cotton growth stage (**T**) and their interaction on the abundance of AOB and AOA, and N_2O-reducing bacteria. Significant effects are in bold (P<0.05). Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought. AOB = Ammonia-oxidizing bacteria, AOA = Ammonia-oxidizing archaea, N_2O = Nitrous oxide.

Factor	**AOB *amoA***		**AOA *amoA***		***nosZ***	
	Post-WL	**Post-PD**	**Post-WL**	**Post-PD**	**Post-WL**	**Post-PD**
N	**<0.001**	**<0.001**	0.650	0.541	**<0.001**	**<0.001**
W	0.176	**<0.001**	0.875	0.720	**<0.001**	**<0.001**
N x W	0.973	**<0.001**	0.964	0.919	**<0.001**	**0.001**
T	**0.038**	**0.025**	0.055	0.075	**<0.001**	**<0.001**
N x T	**0.003**	0.231	0.627	0.710	0.096	0.722
W x T	0.823	0.502	0.914	0.917	0.368	0.914
N x W x T	0.969	0.094	0.999	1.000	0.597	0.858

4.3.2.4.4 Microbial community structure

AOB, AOA and N_2O-reducing bacteria community structures were investigated by terminal restriction fragment length polymorphism (TRFLP) for samples collected at the early squaring, early flowering stages, and final harvest.

TRFLP analysis of AOB generated 4 different TRFs across all soil water treatments and N-fertilizer rates, with three dominant TRFs including TRF-55, 149 and 251. At the early squaring stage, the dominant TRF-251 significantly increased when the amount of N-addition increased from 0 to 300 kg N/ha for all soil water treatments. For Post-PD soils, only three TRFs including TRF-55, 149 and 251 were detected (**Figure 4.13A**). At the early flowering stage, TRFLPs profile also had four different TRFs across all treatments, in which the dominant TRF-251 also significantly increased when the amount of N-fertilizer addition increased from 0 to 300 kg N/ha (P=0.023). The TRF-229 disappeared for samples collected from Post-PD soils (**Figure 4.13B**). At harvest, the same trend was observed as the early squaring and early flowering stages. Four different TRFs were obtained from TRFLP analysis, in which TRF-251 significantly increased with the increase in N-addition rate (P=0.015). The TRF-229 appeared across all different N-fertilizer rates for control and Post-WL soils, but it was not present in Post-PD samples (**Figure 4.13C**). As mentioned in Chapter 2, AOB TRF-149 and TRF-229 was associated with the *Nitrosospira* cluster.

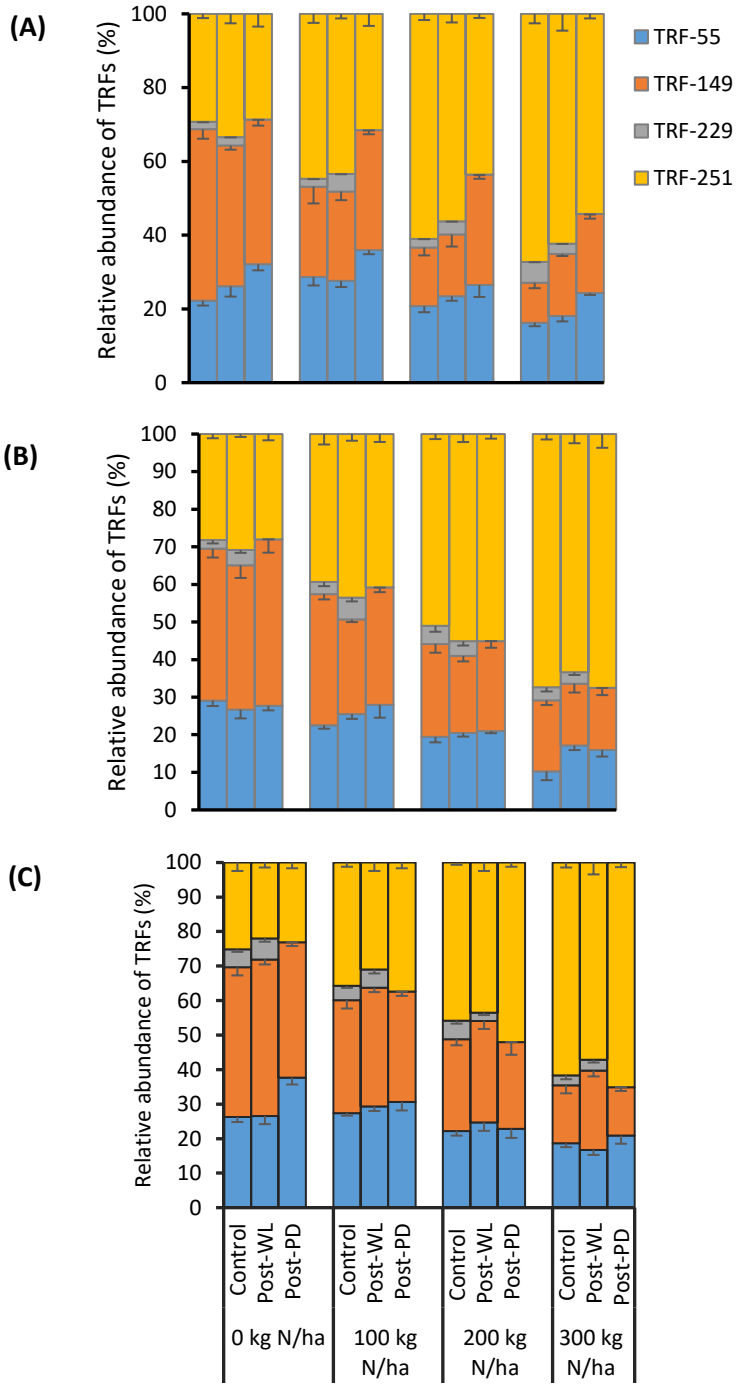

Figure 4.13 Terminal restriction fragment length polymorphism (TRFLP) fingerprints of AOB *amoA* gene fragments across all soil water treatments and N-fertilizer rates at (**A**) the early squaring, (**B**) the early flowering and (**C**) harvest. Values represent mean ± SE (n=4) of each soil water treatment at each N level. Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought. AOB = Ammonia-oxidizing bacteria.

TRFLP analysis of AOA generated eight different TRFs across all treatments, in which four dominant TRFs include TRF-54, 74, 212 and 251. The relative abundance of AOA *amoA* gene fragments did not significantly change when the rate of N-fertilizer addition increased from 0 to 300 kg N/ha for control, Post-WL and Post-PD soils. The unchanged AOA community structures were observed at the early squaring, early flowering and harvest (**Figure 4.14)**. As mentioned in Chapter 2, the phylogenetic affiliation of AOA TRF-54, 74 and 212 could be assigned. Particularly, TRF-54 belonged to *Nitrosopumilus* cluster while TRF-74 and 212 were placed within *Nitrososphaera* cluster.

TRFLP profile of *nosZ* gene fragments showed eight different TRFs across all treatments in which there were five dominant TRFs including TRF-56, 72, 105, 107 and 476. At the early squaring stage, the relative abundance of TRF-107 significantly increased in control and Post-WL samples (P=0.021 and P=0.011) with the increasing N-fertilizer rates whereas both TRF-107 and 476 significantly increased with the increase of N-addition amount for Post-PD soils (P=0.006, P=0.012) (**Figure 4.15A**). At the early flowering stage and harvest, the same trends were observed, particularly the significant increase in the relative abundance of TRF-107 and 476 when N-addition increased (**Figure 4.15B& C**).

Variations in AOB and AOA community structure were examined by principal coordinate analysis (PCO). At each developmental stage, there was clear separation in AOB communities between control and Post-PD soils while control and Post-WL samples were close to each other (**Figure 4.16**). Prolonged-drought event established a legacy effect on soil AOB community (P=0.001). Although PERMANOVA showed a significant effect of previous waterlogging events on AOB community (P=0.001), the separation of AOB communities between control and Post-WL soils was weak. PERMANOVA also showed significant effects of N-addition, growth stage, soil water treatments (prolonged-drought and waterlogging), and their interactions on AOB communities (P<0.05) (**Table 4.12**).

The AOA community structure analysis by PCO did not show clear separation between treatments across all developmental stages. Prolonged-drought and waterlogging had no legacy effects on AOA communities (P=0.057; P=0.321, respectively). Growth stage

significantly affected AOA communities (*P*=0.001), and the interaction of N-addition and growth stage has significant effects on AOA community of Post-WL soils (*P*=0.001). Additionally, PERMANOVA showed a significant effect of N-addition on AOA community of Post-PD soils (*P*=0.012) despite no clear separation between treatments (**Figure 4.17 & Table 4.12**).

PCO analysis showed clear separation of *nosZ*-carrying community structure across all soil water treatments and N-addition rates at each developmental stage (**Figure 4.18**). PERMANOVA showed that prolonged drought and waterlogging established legacy effects on the N_2O-reducing community (*P*=0.001). Additionally, N-addition, growth stage, soil water treatments (prolonged-drought and waterlogging), and their interactions significantly affected the N_2O-reducing community (*P*=0.001) (**Table 4.12**).

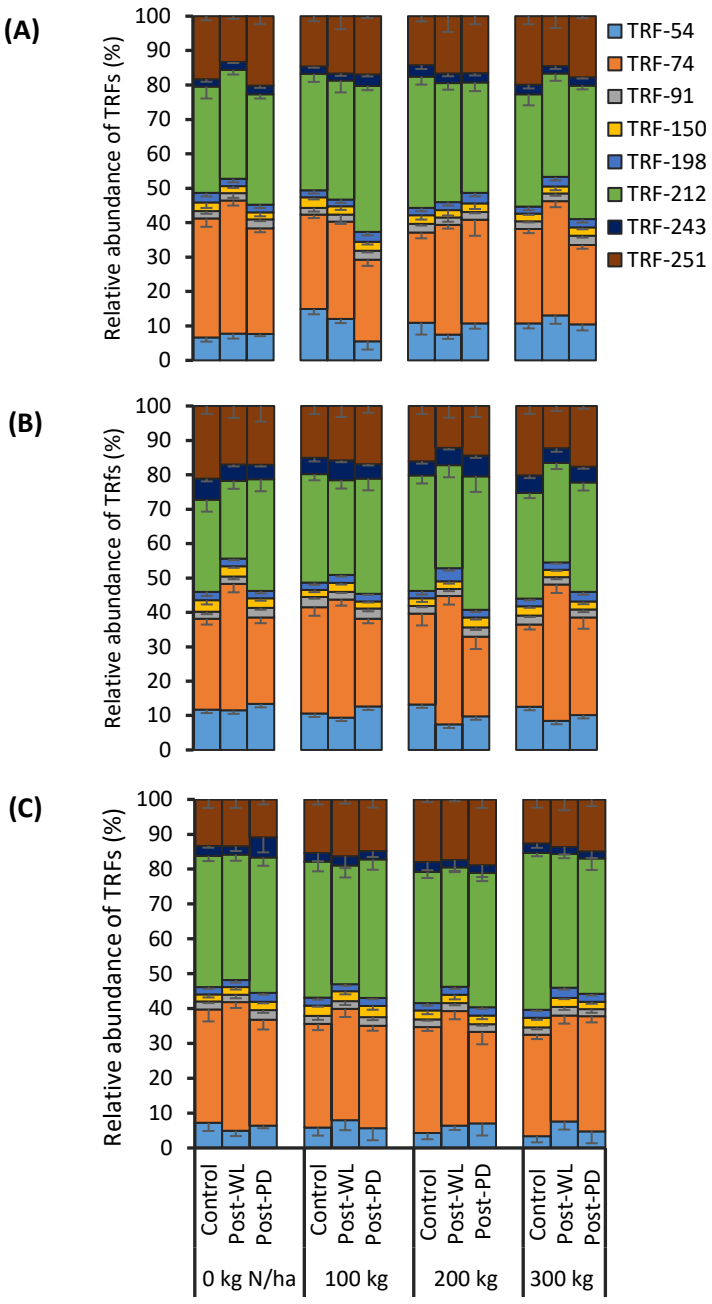

Figure 4.14 Terminal restriction fragment length polymorphism (TRFLP) fingerprints of AOA *amoA* gene fragments across all soil water treatments and N-fertilizer rates at (**A**) the early squaring, (**B**) the early flowering, and (**C**) harvest. Values represent mean ± SE (n=4) of each soil water treatment at each N level. Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought. AOA = Ammonia-oxidizing archaea.

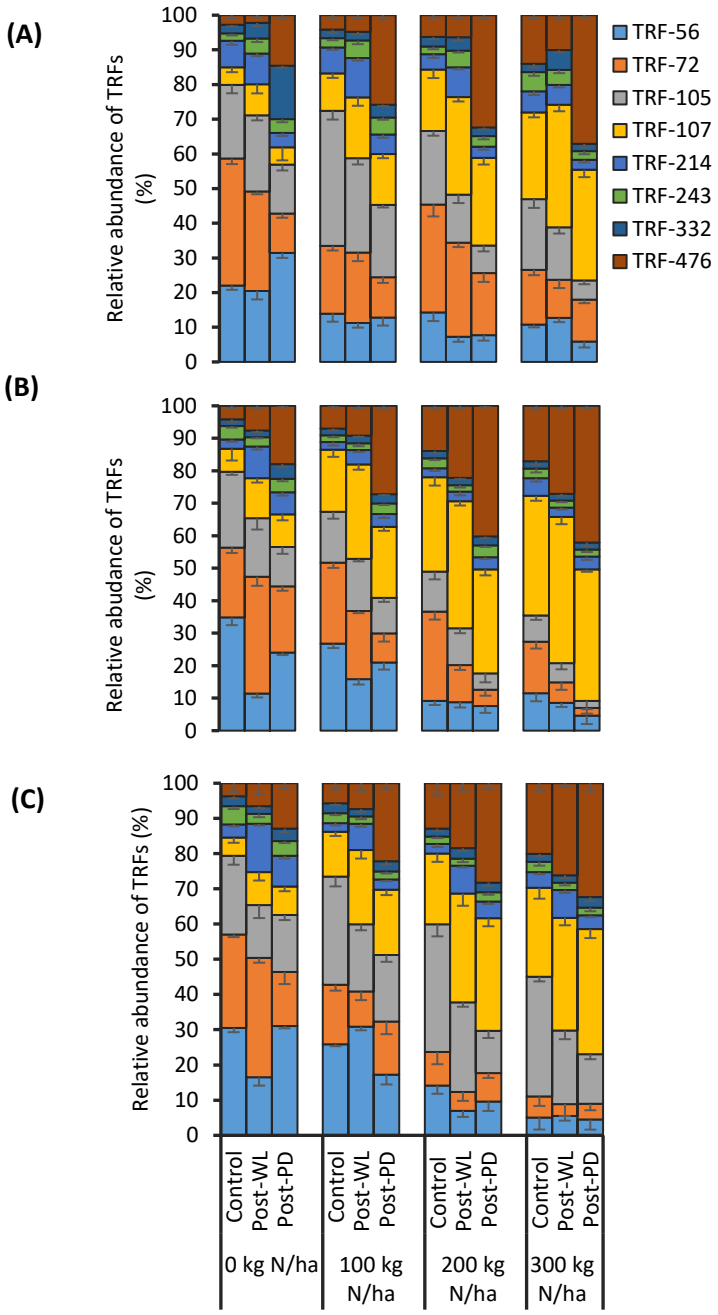

Figure 4.15 Terminal restriction fragment length polymorphism (TRFLP) fingerprints of *nosZ* gene fragments across all soil water treatments and N-fertilizer rates at (**A**) the early squaring, (**B**) the early flowering, and (**C**) harvest. Values represent mean ± SE (n=4) of each soil water treatment at each N level. Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought. N_2O = Nitrous oxide.

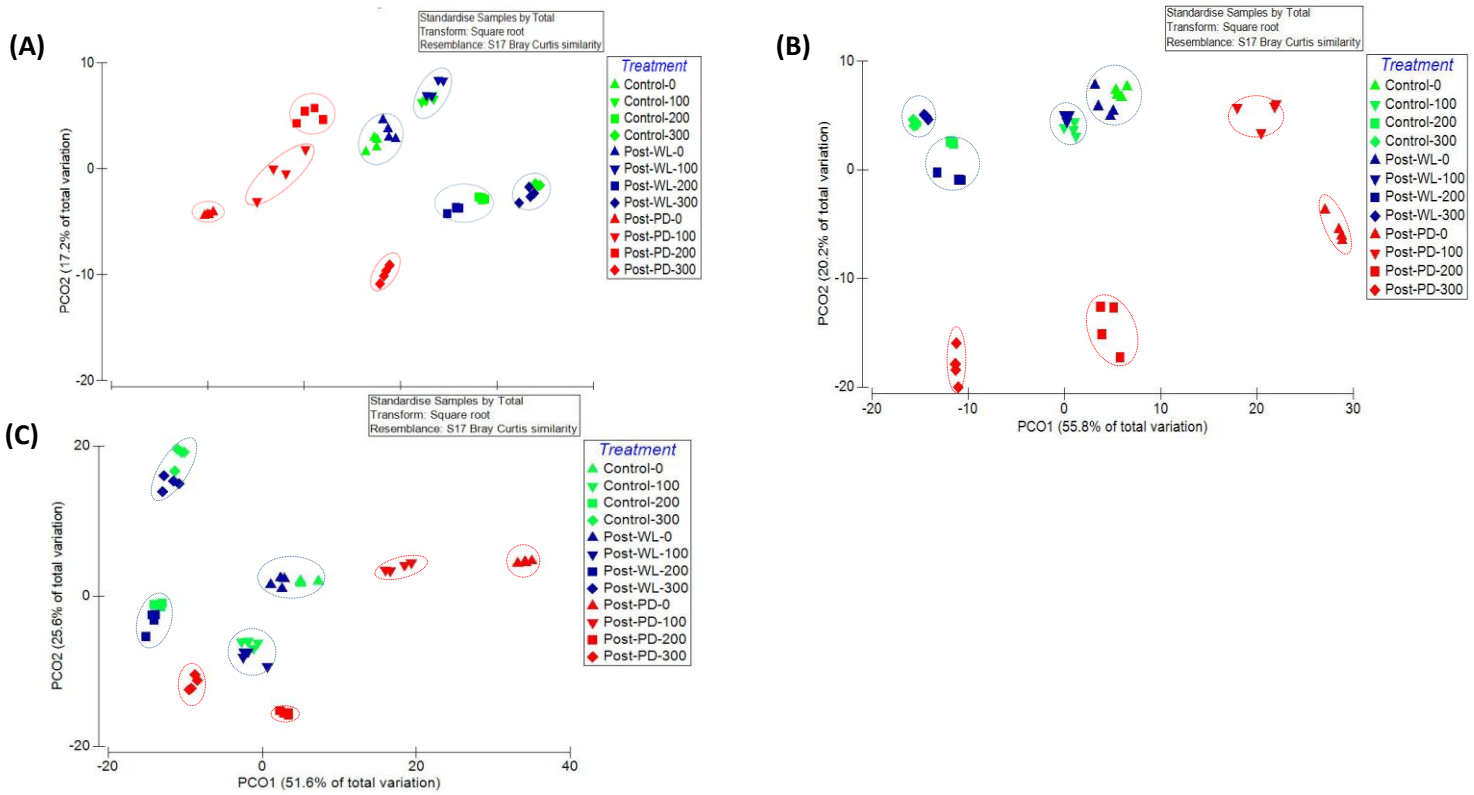

Figure 4.16 Principal coordinates analysis (PCO) derived from the Bray-Curtis dissimilarity matrices showing differences in AOB community structure among different N rate treatments. Data are presented for samples collected at (**A**) the early squaring stage, (**B**) the early flowering stage, and (**C**) harvest. Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought. AOB = Ammonia-oxidizing bacteria.

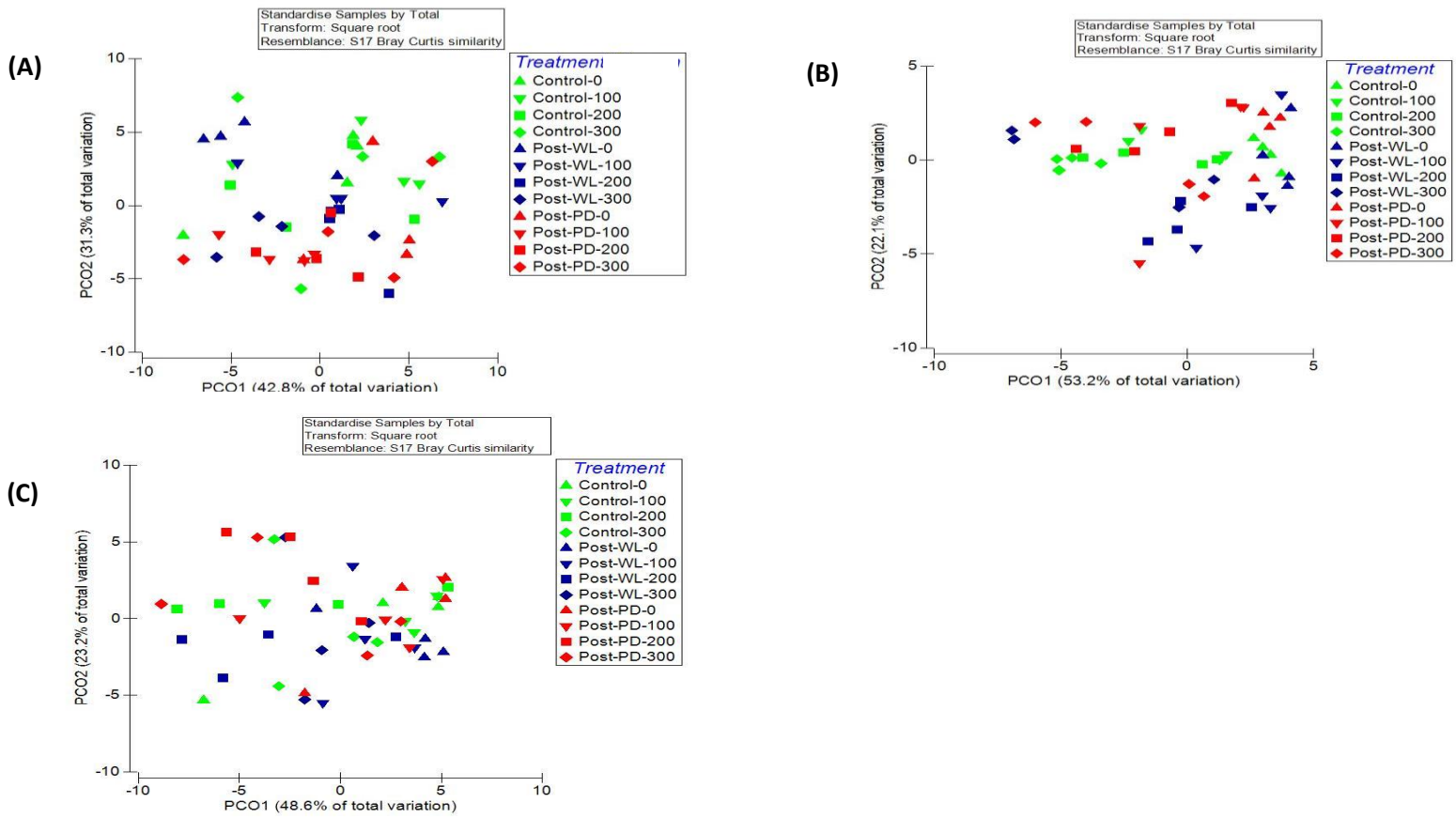

Figure 4.17 Principal coordinates analysis (PCO) derived from the Bray-Curtis dissimilarity matrices showing differences in AOA community structure among different N rate treatments. Data are presented for samples collected at (**A**) the early squaring stage, (**B**) the early flowering stage, and (**C**) harvest. Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought. AOA = Ammonia-oxidizing archaea.

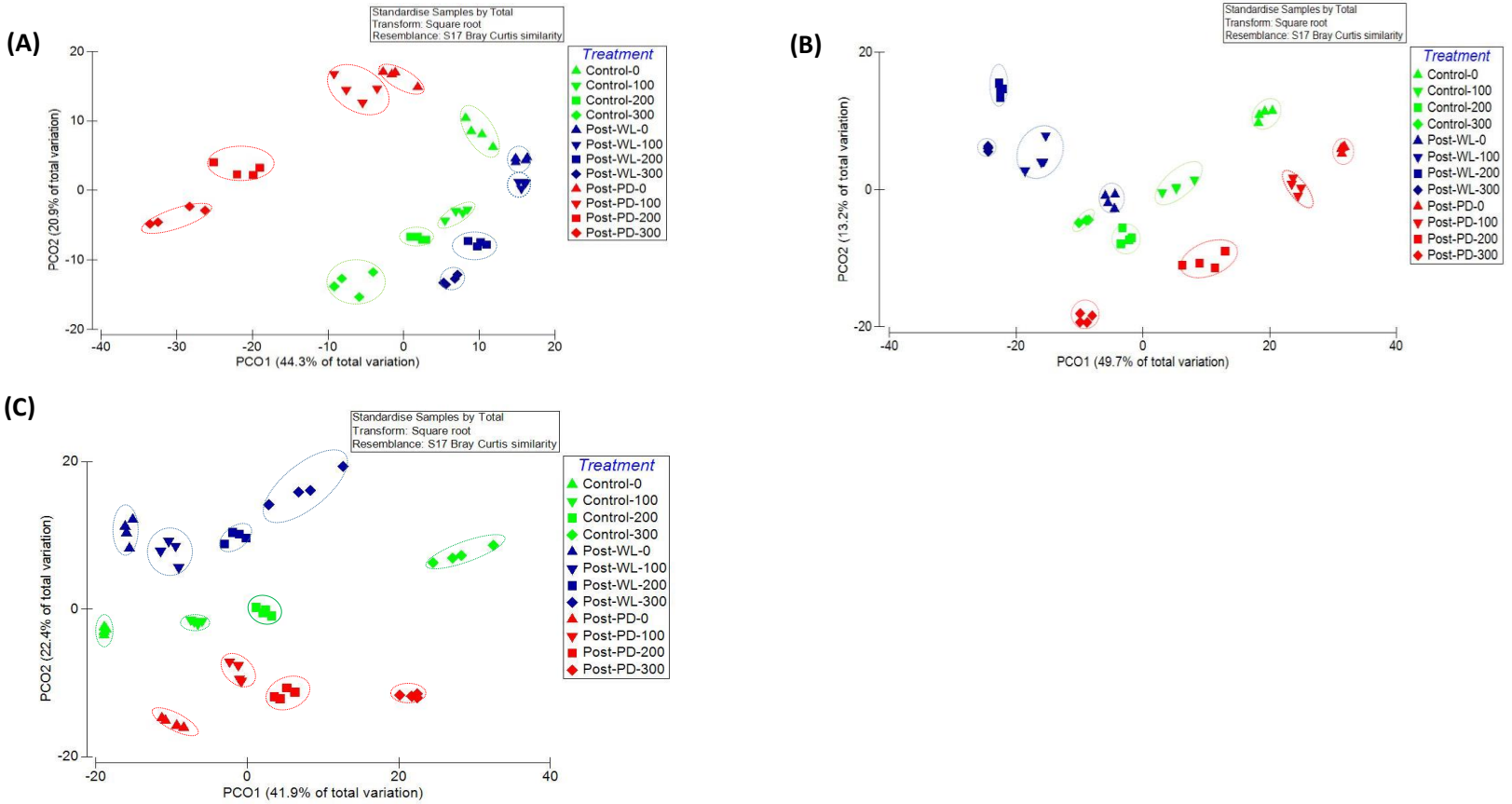

Figure 4.18 Principal coordinates analysis (PCO) derived from the Bray-Curtis dissimilarity matrices showing differences in N_2O-reducing bacterial community structure among different N rate treatments. Data were presented for samples collected at (**A**) the early squaring stage, (**B**) the early flowering stage, and (**C**) harvest. Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought. N_2O = Nitrous oxide.

Table 4.12 Results from PERMANOVA testing for the effects of N-addition (**N**), soil water treatment legacy (**W**) and cotton growth stage (**T**) on (**a**) AOB, (**b**) AOA, and (**c**) NO_2-reducing bacterial structures using Type III sums of squares based 999 permutations of residual under a reduced model. Significant effects are in bold (P<0.05). SS = The sum of square, MS = The mean sum of squares, F = The F-statistic, P = The P-value.

Analysis	**SS**		**MS**		***F***		***P***	
	Post-WL	**Post-PD**	**Post-WL**	**Post-PD**	**Post-WL**	**Post-PD**	**Post-WL**	**Post-PD**
(a)AOB								
N	3570.9	9134	1190.3	3044.7	237.57	489.75	**0.001**	**0.001**
W	35.46	5409	35.46	5409	7.0774	870.06	**0.001**	**0.001**
N x W	35.842	2443.7	11.947	814.56	2.3846	131.03	**0.017**	**0.001**
T	1764.8	3504.3	882.42	1752.2	176.12	281.84	**0.001**	**0.001**
N x T	3816.2	3525.5	636.03	587.58	126.94	94.515	**0.001**	**0.001**
W x T	37.506	1404.4	18.753	702.21	3.7429	112.95	**0.003**	**0.001**
N x W x T	336.37	2930.1	56.061	488.35	11.189	78.554	**0.001**	**0.001**

Table 4.12 (Cont.)

Analysis	SS		MS		*F*		*P*	
	Post-WL	Post-PD	Post-WL	Post-PD	Post-WL	Post-PD	Post-WL	Post-PD
(b) AOA								
N	135.27	194.7	45.091	64.901	1.6382	2.1844	0.079	**0.012**
W	31.377	69.929	31.377	69.929	1.1399	2.3536	0.321	0.057
N x W	64.496	34.888	21.499	11.629	0.78108	0.3914	0.689	0.972
T	14861	14239	7430.5	7119.3	269.96	239.61	**0.001**	**0.001**
N x T	457.67	271.78	76.279	45.297	2.7713	1.5246	**0.001**	0.054
W x T	60.741	113.44	30.371	56.719	1.1034	1.909	0.359	0.044
N x W x T	165.64	134.27	27.607	22.378	1.003	0.75317	0.468	0.794
(c) *nosZ*								
N	4971.2	4355.5	1657.1	1451.8	95.083	83.681	**0.001**	**0.001**
W	3761	3460.2	3761	3460.2	215.8	199.44	**0.001**	**0.001**
N x W	2336.2	2345.1	778.74	781.69	44.684	45.055	**0.001**	**0.001**
T	9671.2	10489	4835.6	5244.5	277.47	302.28	**0.001**	**0.001**
N x T	12524	19914	2087.3	3319.1	119.77	191.3	**0.001**	**0.001**
W x T	4328.7	4992.3	2164.4	2496.1	124.19	143.87	**0.001**	**0.001**
N x W x T	7555	5897.3	1259.1	982.89	72.25	56.652	**0.001**	**0.001**

4.3.2.5 Correlation analyses

The relationship among soil physicochemical properties, soil process rates, plant and molecular measurements were examined by Spearman's rank correlation analysis. There were strong correlations between PNR and AOB *amoA* gene abundance, PDR and *nosZ* gene abundance which were statistically significant (r_S=0.8, P<0.001, and r_S=0.591, P<0.001, respectively) (**Figures 4.19A & 4.20F**). In contrast, no significant correlation between AOA *amoA* gene abundance and PNR was observed (r_S=0.159, P=0.058) (**Figure 4.19B**). Stepwise regress analysis was used to reveal the mechanism which was the best predictor for PNR, PDR, NMR, AOB, AOA and *nosZ* gene abundance (**Table 4.13**). AOB abundance and total N were strongly correlated with PNR and NMR, respectively. PDR was strongly correlated with soil pH, inorganic N, total N, and *nosZ* gene abundance. Soil NH_4^+ content was responsible for changes in AOB and AOA abundance while total N was responsible for changes in *nosZ* gene abundance (**Table 4.13**).

In this study, plant measurements included plant height and node, leaf and boll number, leaf area, boll size, leaf, stem and root dry mass, total dry mass, seed C and N, and leaf, stem and root N. In terms of plant productivity, there were strong significant correlations between seed cotton yield and AOB *amoA* gene abundance, PNR and NMR (**Figure 4.19 C&E and Figure 4.20D**). Stepwise regression analysis was carried out to determine the best predictor for each plant variable (**Table 4.14**).

The first five PCOs explained 92.6% of the variation in the AOB TRFLP pattern. AOB community structure along PCO1, PCO2 and PCO4 was significantly correlated with PNR (r_S=-0.759, P<0.001; r_S=0.231, P=0.006; and r_S=-0.226, P=0.007) (**Table 4.15**). In contrast, despite 98.51% of the variation in the AOA TRFLP patterns explained by the first five PCOs, no significant correlation between PNR and AOA community structure was observed (P>0.05) (**Table 4.15**). Regarding *nosZ* gene, the first five PCOs explained 75.9% of the variation in the TRFLP profile of NO_2-reducing community and only *nosZ*-hosting community along PCO3 was significantly correlated with PDR (r_S=0.515, P<0.001) (**Table 4.15)**.

Table 4.13 Variable responsible for the changes in soil process rates and the functional microbial communities. AOB = Ammonia-oxidizing bacteria, AOA = Ammonia-oxidizing archaea, N_2O = Nitrous oxide. PDR = Potential denitrification, PNR = Potential nitrification rate, MMR = Net N mineralization rate. NH_4^+ = Ammonium, NO_3^- = Nitrate, N = Nitrogen. R^2 = The coefficient of determination, *F* = The *F*-value, *P* = The *P*-value.

Soil measurements	**Result**	***R²***	***F***	***P***
PNR	Y=0.406 + 0.042 (AOB abundance)	0.628	239.786	**<0.001**
NMR	Y= -0.057 +0.494 (soil total N content)	0.623	76.135	**<0.001**
PDR	Y= 0.618 - 0.021 (soil NH_4^+ content) +0.001 (soil NO_3^- content)-0.084 (pH) +0.001 (*nosZ* abundance) +0.22 (soil total N content)	0.773	134.7	**<0.001**
AOB *amoA*	Y= -2.177 + 14.811 (soil NH_4^+ content)	0.628	239.786	**<0.001**
AOA *amoA*	Y= 173.802 + 6.464 (soil NH_4^+ content)	0.089	13.864	**<0.001**
nosZ	Y= 4.845 + 43.468 (soil total N content)	0.515	74.762	**<0.001**

Table 4.14 Variable responsible for the changes in the plant growth and productivity measurements. NH_4^+ = Ammonium, NO_3^- = Nitrate, N = Nitrogen. AOB = Ammonia-oxidizing bacteria. PNR = Potential nitrification rate, MMR = Net N mineralization rate. R^2 = The coefficient of determination, *F* = The *F*-statistic, *P* = The *P*-value.

Plant measurement	**Result**	***R²***	***F***	***P***
Leaf number	Y=8.441-6.676 (soil NH_4^+ content) + 16.298 (PNR) + 2.239 (soil NO_3^- content)	0.886	271.456	**<0.001**
Leaf area	Y= -642.717 + 136.114 (soil NO_3^- content) + 2869.498 (total N content)	0.848	255.684	**<0.001**
Boll number	Y= 0.639 + 1.633 (soil NO_3^- content)	0.815	202.488	**<0.001**
Boll size	Y=5.174 + 6.697 (soil total N content)	0.659	88.965	**<0.001**
Leaf dry mass	Y= 1.027 + 7.06 (soil total N content) + 0.692 (soil NO_3^- content)	0.919	524.73	**<0.001**
Stem dry mass	Y= 0.365 - 13.269 (NMR) + 0.62 (soil NO_3^- content) + 29.202 (soil total N content)	0.853	265.952	**<0.001**
Root dry mass	Y= -19.595 + 2.219 (soil NO_3^- content) + 3.492 (AOB abundance) + 0.192 (PNR)	0.838	237.711	**<0.001**
Seed cotton yield	Y= -5.195 + 9.889 (PNR) + 23.984 (soil total N content) +1.523 (soil NO_3^- content)	0.943	240.932	**<0.001**

Total dry biomass	Y= -2.276 + 16.48 (PNR) + 56.153 (soil total N content) + 2.923 (soil NO_3^- content)	0.939	709.074	**<0.001**
Plant height	Y= 9.505 - 3.278 (soil NH_4^+ content) + 51.126 (soil total N content) - 0.08 (soil NO_3^- content)	0.741	99.628	**<0.001**
Plant node	Y= 2.164 + 14.864 (soil total N content) -1.194 (soil NH_4^+ content) + 1.858 (PNR)	0.594	68.363	**<0.001**
Seed N	Y= 9.613 + 21.914 (soil total N content)	0.800	213.299	**<0.001**
Seed C	Y=506.05 + 27.525 (soil total N content)	0.378	27.955	**0.001**
Leaf N	Y= 7.079 + 7.529 (soil total N content)	0.603	69.887	**<0.001**
Stem N	Y= 6.792 + 0.112 (soil NO_3^- content)	0.430	34.679	**<0.001**
Root N	Y= 4.794 + 0.065 (soil NO_3^- content)	0.260	16.203	**<0.001**

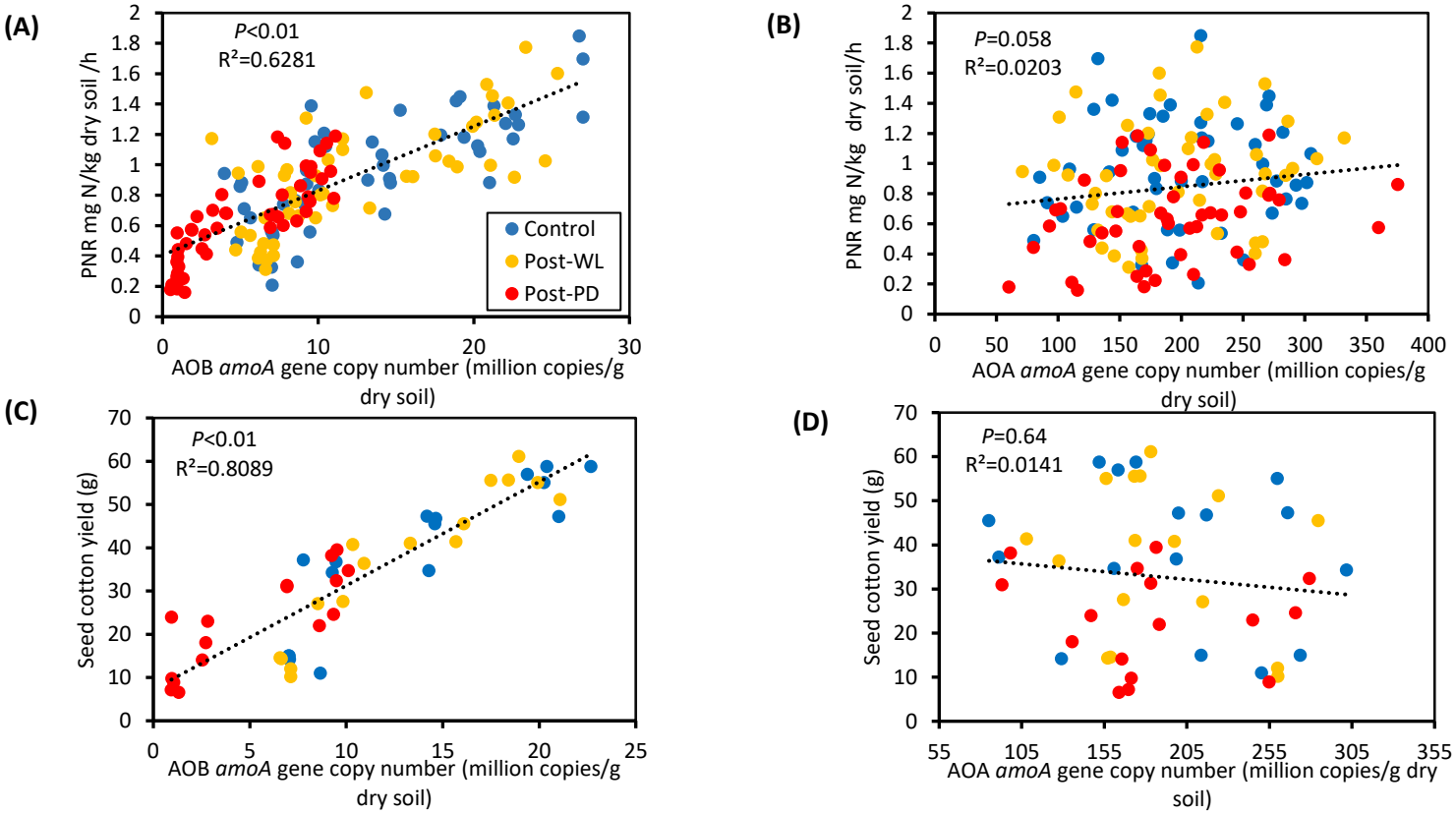

Figure 4.19 Relationships between variables including the abundance of (**A**) AOB, (**B**) AOA and PNR; (**C**) seed cotton yield and the abundance of (**C**) AOB and **(D)** AOA; PNR and **(E)** seed cotton yield and **(F)** total biomass; total biomass and the abundance of **(G)** AOB and **(H)** AOA. Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought. AOB = Ammonia-oxidizing bacteria, AOA = Ammonia-oxidizing-archaea, PNR = Potential nitrification rate.

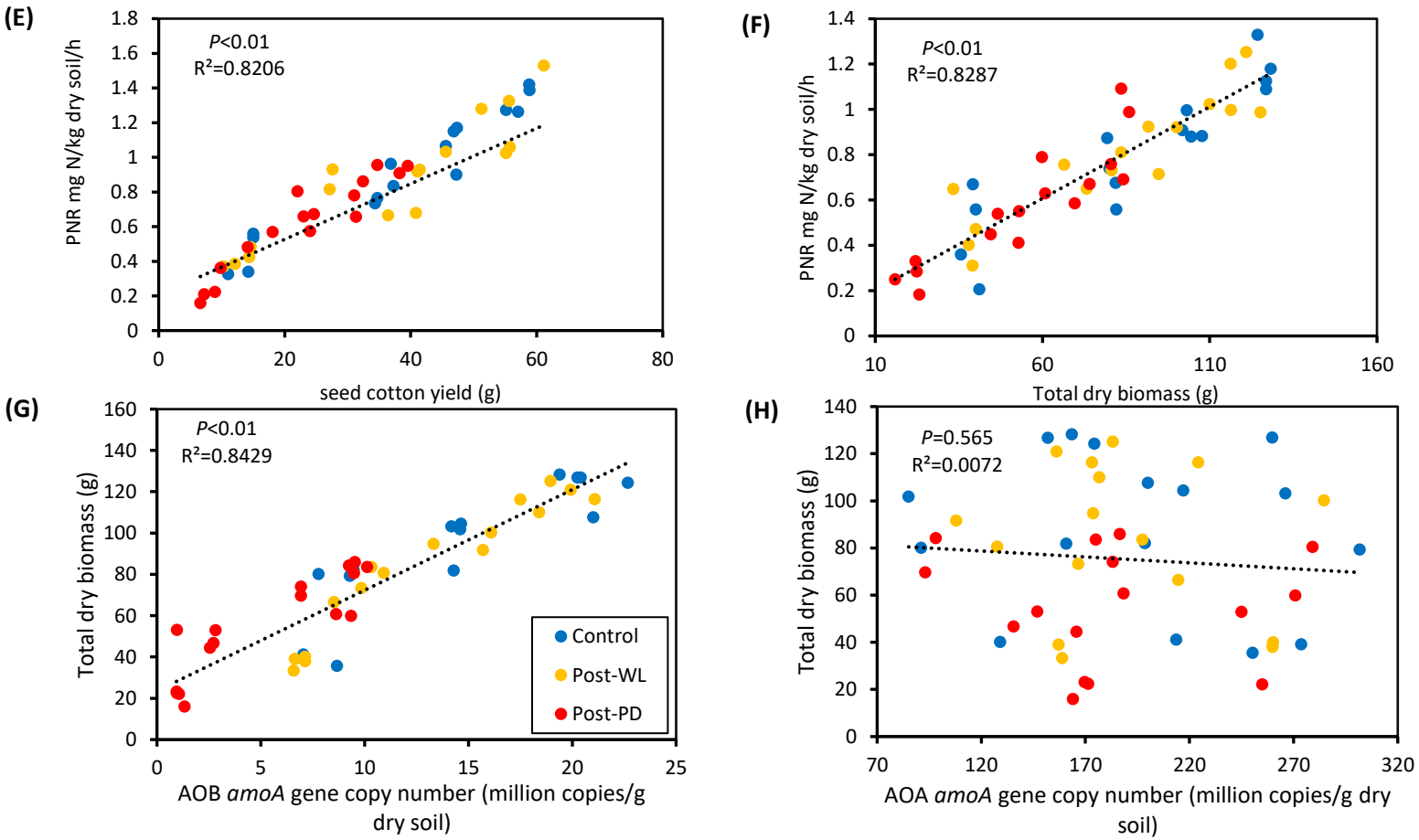

Figure 4.19 (Cont.)

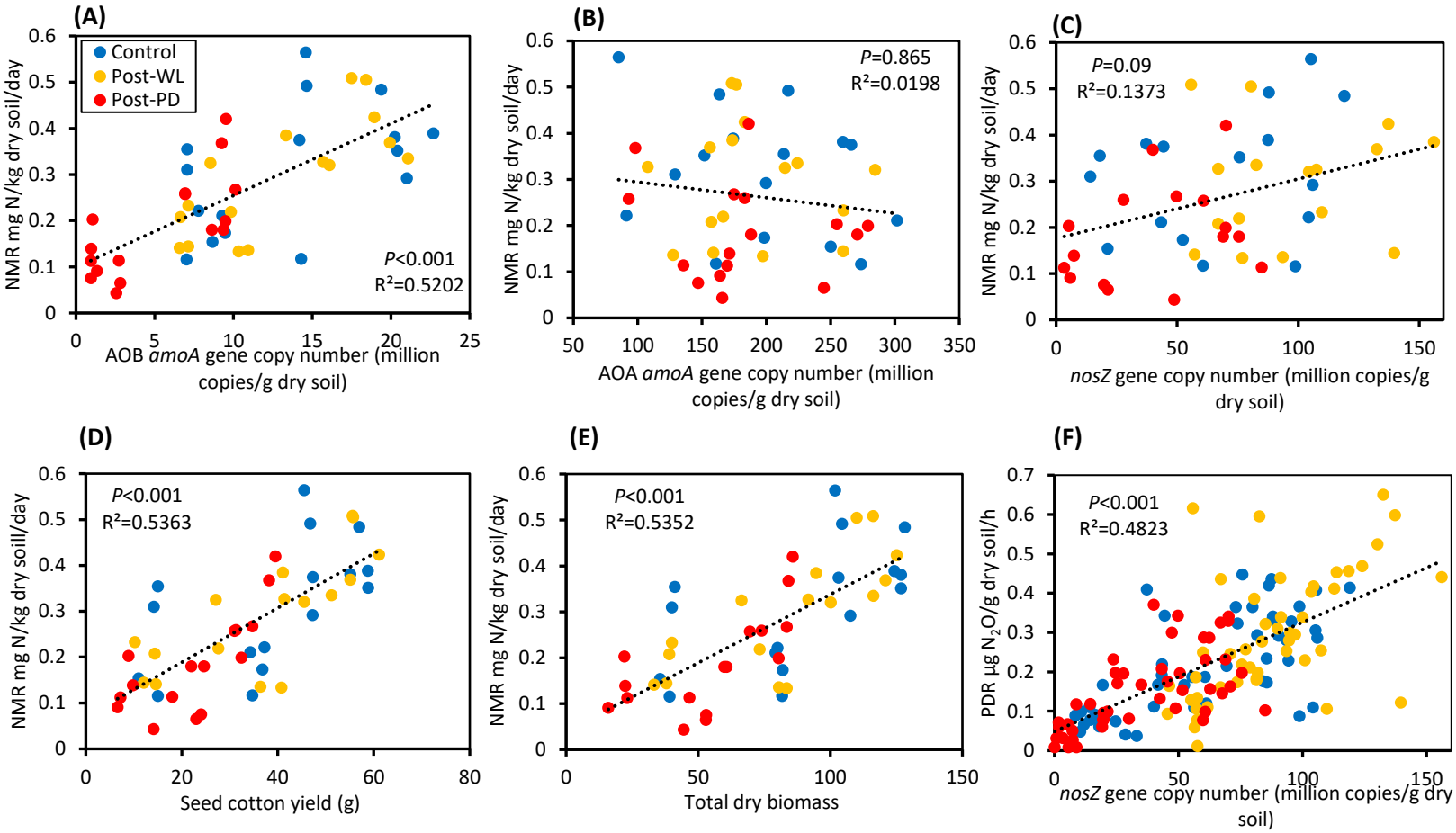

Figure 4.20 Relationships between NMR and **(A)** AOB *amoA*, **(B)** AOA *amoA* and **(C)** *nosZ* gene abundance; NMR and **(D)** seed cotton yield and **(E)** total dry biomass; **(F)** PDR and the abundance of *nosZ* gene. NMR = Net N mineralization, PDR = Potential denitrification rate. AOB = Ammonia-oxidizing bacteria, AOA = Ammonia-oxidizing archaea.

Table 4.15 The variation explained, the Spearman rank correlation coefficient (r_S), and the *P* values of correlations between potential nitrification rate (PNR), net N mineralization rate (NMR), potential denitrification rate (PDR) and (**a** & **b**) AOB, AOA compositions and (**c**) NO_2-reducing bacterial composition, as summarized by the top five principal coordinates. Significant effects are in bold (*P*<0.05). AOB = Ammonia-oxidizing bacteria, AOA = Ammonia-oxidizing archaea, N_2O = Nitrous oxide.

Statistic	PCO1	PCO2	PCO3	PCO4	PCO5
(a) AOB					
Variation explained (%)	53.4	14.9	10.3	8.3	5.7
PNR					
r_S	**-0.759**	**0.231**	-0.025	**-0.226**	0.056
P	**<0.001**	**0.006**	0.77	**0.007**	0.056
NMR					
r_S	**-0.478**	0.071	-0.204	-0.057	0.2
P	**0.01**	0.632	0.165	0.699	0.173
(b) AOA					
Variation explained (%)	79.9	9.19	6.07	1.75	1.6
PNR					
r_S	0.201	-0.205	-0.333	-0.142	0.057
P	0.062	0.054	0.051	0.09	-.499
NMR					
r_S	-0.366	-0.079	-0.275	0.276	0.185
P	0.054	0.594	0.059	0.066	0.109
(c) *nosZ*					
Variation explained (%)	35	17.9	9.43	7.65	5.92
PDR					
r_S	0.146	0.139	**0.515**	0.102	0.013
P	0.061	0.097	**<0.001**	0.222	0.879
NMR					
r_S	0.341	0.273	0.084	-0.134	-0.017
P	0.085	0.061	0.569	0.365	0.907

4.4 Discussion

My study found that soil exposed to prolonged-drought prior to planting negatively affected soil physicochemical properties, microbial communities, and their functions, thereby ultimately reducing plant growth and crop productivity. In particular, soil N availability was significantly reduced. Although the rate of N-addition up to 300 kg N/ha was used, the legacy effects of prolonged-drought on soil characteristics, plant growth and crop productivity could not be diminished. In contrast to prolonged-drought, waterlogging event posed legacy effects only on *nosZ*-carrying communities and potential denitrification rate, resulting in no significant differences in plant growth and crop productivity when compared to the control. In this study, the responses of AOB and N_2O-reducing bacterial communities to legacy effects of extreme weather events and N fertilizer addition, and strong correlations between these microbial communities and their functions provide insights into role of soil microbial communities in maintaining process rates under environmental disturbance.

4.4.1 Effects of prolonged-drought and waterlogging events prior to planting and different rates of N fertilizer addition on soil and plant

Soil physicochemical properties, microbial communities, and their functions

Soil moisture content is a key factor which contributes to patterns of N and C flows, and structures microbial communities and activities in soils (Gleeson *et al.*, 2010, Barnard *et al.*, 2013). Therefore, changes in the moisture content of soils exposed to extreme weather events including pro-longed drought and waterlogging may result in a legacy effect on soil properties. Soil with a legacy of historical conditions prior to planting can generate permanent alterations in microbial community structure and function (Fraterrigo *et al.*, 2006, Strickland *et al.*, 2009), and consequently affecting crop yields.

In my study, prolonged-drought condition was simulated by air-drying soil for 4 months until soil moisture did not change any more. Soil moisture content dropped to 6.2% after nearly two months of air-drying. The moisture condition persisted for approximately two

months, significantly affecting soil microbial communities and their functions at pre-sowing, indicating the vulnerability of soil functional communities in the irrigated cotton farming to dry and rewetting stress. Although soil bacteria may undergo recovery upon rewetting (Fierer & Schimel, 2003, Göransson *et al.*, 2013), the abundance of AOB and N_2O-reducing bacteria and soil process rates (including potential nitrification, denitrification and N mineralization rates) were significantly lower than those of control soils, indicating significant loss of drought sensitive bacteria. In support, previous studies have shown severe consequences of drought on soil microbial community structure and activity (Van Meeteren *et al.*, 2008, Hueso *et al.*, 2012). These effects may be attributed to a reduction of key substrate diffusion rates and bacterial mobility to access soluble nutrients when soil was exposed to drought (Van Meeteren *et al.*, 2008, Bastida *et al.*, 2006). The drought-induced soluble salt concentration may also explain for the depleted soil microbial communities (Geng *et al.*, 2014).

Regarding ammonia-oxidizing communities, prolonged-drought and re-wetting significantly affected soil AOB, but not AOA. This may be due to strong physiological counteractions of AOA which are known to inherently resist to water or other stresses whereas AOB are Gram-negative bacteria and are much more sensitive than AOA in response to stresses (He *et al.*, 2012, Zhang *et al.*, 2012a). Specifically, during air-drying soils, the substrate limitations and reduced microbial mobility did not affect the AOA survival possibly due to the significantly high affinity for substrate of AOA (Martens-Habbena *et al.*, 2009). Additionally, the AOA cell size is generally 10-fold smaller than the cell size of AOB, and hence increasing the capacity of AOA against stresses due to the increased surface-to-volume (Martens-Habbena & Stahl, 2010). The nitrification pathways of AOA may be much more energy-efficient than that of AOB (Könneke *et al.*, 2014).

Re-wetting dry soils has been well-known to generate a pulse of N mineralization, resulting in an increase in soil substrate availability (Schimel *et al.*, 2011, Göransson *et al.*, 2013). Such increases in soil substrate availability can affect AOB and AOA (Thion &

Prosser, 2014), however, in this study, I provide evidence that even when normal moisture conditions were returned, legacy impacts of drought remained for AOB, but not for AOA, during approximately 6 months of the experiment operation. This suggests that AOB community in the irrigated cotton farming was highly vulnerable to extreme drought event and they may take a long time to recover.

Prolonged-drought before planting, established a legacy effect on soil physiochemical properties, soil processes, and functional microbial community structure and abundance, particularly AOB and NO_2-reducing communities, and subsequent consequences for crop growth and productivity. Previous studies have also provided evidence of drought legacy impacts on soil nutrients, microbial community and activities, and consequences for plant growth (Banerjee *et al.*, 2016, Cavagnaro, 2016, Meisner *et al.*, 2013a). To recover depleted soil systems due to environmental disturbances, N fertilizers may be used to improve soil fertility and crop yields (Bruulsema *et al.*, 2004, Boquet *et al.*, 2009, Lu *et al.*, 2015). In my study, N-addition up to the rate of 300 kg N/ha could not counteract the legacy of prolonged drought event prior to pre-sowing. However, N-addition significantly affected soil processes and functional microbial communities, particularly increased N mineralization and nitrification rates, and AOB, *nosZ*-carrying community abundance and shifts in AOB and *nosZ*-carrying community structure. Thus, this confirmed the long-lasting legacy effects of prolonged-drought on soil properties, especially AOB community in irrigated cotton farming. The positive response of N mineralization to N fertilizer addition may be attributed to increases in soil nutrition (Delin & Lindén, 2002, Kadono *et al.*, 2008), readily mineralizable soil organic N and microbial activities (Aggangan *et al.*, 1998). The higher plant biomass obtained with the addition of N fertilizer also explains the reason that N supply stimulates net N mineralization (Zhang *et al.*, 2012b). The increased N mineralization rates in response to inorganic N fertilizer addition were also observed in other studies (Chen *et al.*, 2011, Schröder *et al.*, 2000, Loecke *et al.*, 2012, Zhang *et al.*, 2015). The abundance of AOB and *nosZ*-carrying community were significantly correlated with soil NH_4^+ and total N content, suggesting that N-addition and the legacy effects of prolonged-drought changed

microbial abundance by affecting soil physicochemical properties; in particular, N-addition x Legacy effect of prolonged-drought significantly influenced soil NH_4^+ and total N.

My study did not find legacy effects of waterlogging on soil nutrients and ammonia-oxidizers. The diffusion rate of O_2 in soil environment decreased approximately 10,000-fold upon waterlogging, resulting in hypoxia or even anoxia which can inhibit nitrification and facilitate denitrification (Tiedje, 1988, Wegner, 2010). The community structure and abundance of ammonia-oxidizers of Post-WL soils at pre-planting did not significantly differ from that of control soils, suggesting a resilience of these microorganisms after waterlogging stress although I observed significant effects of waterlogging events on ammonia-oxidizing communities in the experimental Chapter 2. Few previous studies have reported soil processes and biota affected by a history of rainfall event or high soil water content prior to planting, and hence impact plant growth (Cavagnaro, 2016, Meisner *et al.*, 2013b). In this study, I provided contrasting evidence, particularly the recovery of soil processes and nitrifiers after waterlogging events, and thus crop productivity remained unchanged. These results suggest that soil microbes and processes may be resilient to waterlogging stress prior to planting, thereby maintaining crop yields in subsequent years in irrigated cotton farming.

Regarding *nosZ*-carrying community, the higher *nosZ* gene abundance and differences in community structure were detected before planting in Post-WL soils. Other studies also found markedly increases of *nosZ* abundance after soil exposed to waterlogging (Uchida *et al.*, 2014, Yang *et al.*, 2016). The legacy effect of waterlogging and N-addition, separately and their interaction, were also observed during the experiment. The *nosZ* gene abundance was significantly correlated with soil total N content. Therefore, it suggests that N-addition and legacy of waterlogging changed the abundance of NO_2-reducing bacteria by affecting soil total N content. The *nosZ*-carrying community responded to N-addition through an increase in *nosZ* gene abundance with the increase in the rate of N-addition in my study. This is consistent with the findings of Hamonts *et*

al. (2013) and Chen *et al.* (2012b) which demonstrated an increase in *nosZ* gene abundance. Although there was strong correlation between PDR and *nosZ* gene abundance, the soil NO_3^- profile and crop productivity were not affected since all soils were aerobic. It suggests that legacy effects of waterlogging on soil inorganic pools may depend on the extent to which soil is aerobic and anaerobic. A strong relationship between PDR and *nosZ* gene abundance also suggests that the abundance of *nosZ*-bearing bacteria contribute to mediation of the denitrification rate in irrigated cotton soil, and any further waterlogging may result in rapid loss of N from the plant-soil systems.

Crop growth and productivity

The legacy effects of prolonged drought prior to planting on plant growth were detectable throughout the experiment. Plant growth and yield were significantly correlated with soil NH_4^+, NO_3^-, total N, the abundance of AOB, *nosZ*-carrying bacteria, PNR and PDR. Since N-addition and legacy of prolonged-drought had been demonstrated to reduce seed cotton yield and total dry mass significantly, it can be concluded that two factors affected these values through changing soil nutrients and microbial communities. Cavagnaro (2016) and Meisner *et al.* (2013b) also found clear legacy effects of dry-rewetting cycles on soil properties and plant growth. However, the interaction between legacy effects and N-addition were not examined in these studies. In my study, N-addition was not able to completely diminish the legacy effects of previous prolonged-drought, but soil nutrient availability and microbial communities were stimulated with the increase in N-addition rates. The highest seed cotton yield was obtained at the rate of 300 kg N/ha in all soil treatments, suggesting that high N-addition can reduce the loss of farm productivity after extreme weather events. This supports my hypothesis of a need for applying higher rates of N-fertilizer if there is an establishment of extreme weather legacy effects. Previous studies have provided the evidence of the interactive effects of soil water regimes and N addition on soils and plants (Grime & Curtis, 1976, Haddad *et al.*, 2002, Zhang *et al.*, 2015, Fiala *et al.*, 2011, Rahimi *et al.*, 2013, Bi *et al.*,

2012, Xu *et al.*, 2015, Krček *et al.*, 2008). For example, Abid *et al.* (2016) reported that N supply potentially alleviated adverse effects of drought stress on wheat productivity whereas Hartmann *et al.* (2013) observed an interaction of drought and N fertilization on soil processes and functional microbial communities in a grassland soil, particularly nitrifiers and denitrifiers, and their associated processes. However, in my study, the interaction of drought legacy effects and N addition on soils and plants were examined, providing novel evidence of soil and crop yield responses to N addition in the context of the establishment of drought legacy impacts.

4.4.2 Effects of N-addition on AOB and AOA communities and their function

In my study, AOB responded significantly to N-fertilizer addition and addition rates. Although AOA outnumbered their counterpart AOB, no obvious effects of N-addition on AOA community were observed. N-addition had significant effects on AOB, but not AOA community structure, and AOB abundance significantly increased with the increase in N-addition rate. This could be attributed to different substrate affinity of AOB and AOA. AOB was found to prefer high concentrations of ammonia (Di *et al.*, 2010), and AOA have higher affinity to ammonia substrate than AOB (Martens-Habbena *et al*., 2009), potentially resulting in differences in their responses to N-addition. The other explanation is that AOA may be heterotrophic or mixotrophic as they can use organic carbon; for example, the isolated archaea strains *Nitrosopumilus maritimus* and *Nitrososphaera viennensis* can assimilate amino acid and pyruvate to serve their growth, respectively (Walker *et al.*, 2010, Tourna *et al.*, 2011). No clear responses of AOA to urea application in my study support this premise. In agreement to this, a number of studies have found greater growth of AOA in response to organic fertilizer addition than to inorganic N (Kelly *et al.*, 2011, Levičnik-Höfferle *et al.*, 2012). The significant increases in AOB, but not AOA, abundance in soils fertilized with high levels of N-fertilizers have also been reported in previous studies of grassland soils (Shen *et al.*, 2008, Di *et al.*, 2009, Chen *et al.*, 2013). My findings contribute to the conclusion of stronger response of AOB rather than AOA to inorganic N fertilizer addition in irrigated cotton soils which is in

agreement with some studies of ammonia-oxidizers in response to N-addition in agricultural soils (Zhou *et al.*, 2014, Shen *et al.*, 2014).

4.4.3 Relationship between functional microbial communities and key soil processes, and consequences for crop productivity

In my study, significantly strong correlations between PNR and AOB abundance and community structure were observed, suggesting that nitrification might be driven principally by AOB in irrigated cotton soils fertilized with different rates of N fertilizer. Similarly, NMR significantly correlated with AOB abundance and community structure, and hence indicated that ammonium availability was a factor shaping the AOB community in irrigated cotton soils. My study is in agreement with a number of previous studies showing a link between NMR and AOB community structure in different ecosystems such as grassland, forest and cropland soils (Malchair *et al.*, 2010, Carney *et al.*, 2004, Malchair & Carnol, 2013, Glaser *et al.*, 2010). Therefore, in the context of legacy effects of prolonged-drought, soil ammonium availability was depleted due to reduced N mineralization rate, resulting in shifts in AOB community and the nitrification process rate. Such changes in soil inorganic N pools substantially influenced cotton crop yields.

Regarding N_2O-reducing community, there were also significant correlations between PDR and *nosZ* gene abundance and community structure along PCO3 despite that this axis explained only 9.43% of the variation in TRFLP profile. My results are in line with the study by Hamonts *et al.* (2013) who examined denitrifying population dynamics and the associated process in waterlogged wheat soils. In my study, despite that no legacy effects of waterlogging on soil nutrient availability was established, further waterlogging will increase in N loss from soils harboring the higher abundance of denitrifying community, thereby potentially reducing crop productivity.

4.5 Conclusions

My study showed strong legacy impacts of prolonged drought, but only marginal legacy impacts of waterlogging on soils and crop yields. This is due to deleterious effects of

prolonged-drought on the functional microbial abundance and community structure, particularly AOB, and soil nutrient availability, thereby negatively influencing the rate of nitrification process, and consequently crop productivity. Surprisingly, the legacy impacts of prolonged drought persisted throughout the experiment although up to 300 kg N/ha N fertilizer was applied, suggesting these depleted microbial communities would take a long time to recover. The only legacy impacts of waterlogging were low NO_3^- levels remained in soils without N-addition, and shifts in the abundance and structure of N_2O-reducing community. It suggests most of soil microbial communities and processes were resilient to legacy effects of waterlogging. However, if further waterlogging occurs, loss of N from the plant-soil systems will be rapid, potentially compromising farm productivity. My findings provide relevant knowledges for developing adaptation and N management strategies to minimize the loss of farm productivity in the context of increased frequency and intensity of extreme weather events. This study only focused on legacy effects of short-term waterlogging and prolonged-drought on soils and crop productivity. Due to the fact that waterlogging may occur in long-term periods under future climatic conditions, further studies should include soils exposed to prolonged waterlogging prior to planting.

CHAPTER 5 RESPONSES OF SOIL BACTERIAL COMMUNITY AND MICROBIAL RESPIRATION TO NITROGEN FERTILIZER REGIMES AFTER EXPOSURE TO EXTREME WEATHER EVENTS: FLOODING AND PRO-LONGED DROUGHT

5.1 Introduction

Extreme weather events including flooding and prolonged-drought alter soil moisture conditions, and thus may affect soil processes, biota, and plant growth in subsequent years (Meisner *et al.*, 2013b). In Chapter 4, the prolonged-drought period prior to planting established strong negative legacy effects on soil physicochemical properties, N processes, and N-cycling communities including AOB and N_2O-reducing bacteria, consequently influencing crop productivity. In contrast, previous waterlogging events only significantly affected the soil NO_3^- and N_2O-reducing community. Given that N fertilizer supply generally enhances soil fertility and crop yields (Selassie, 2015, Nkebiwe *et al.*, 2016), N addition up to 300 kg N/ha could not completely diminish drought legacy effects whereas the legacy effect of waterlogging on soil NO_3^- was counteracted, thereby maintaining crop productivity. The responses of N-cycling microbial communities to legacy effects of extreme weather events and N fertilizer supply (Chapter 4) raised a question: do entire soil bacterial communities similarly respond to N-cycling communities? Previous studies have suggested different vulnerabilities of different soil bacterial groups to drought and flooding stresses (Graff & Conrad, 2005, Schimel *et al.*, 2007, Chodak *et al.*, 2015); however little is known about the taxonomic structure and diversity of soil bacterial communities in response to the legacy effects of extreme weather events. In addition, there were no clear trends for the response of soil total microbial communities to N fertilizer addition (Lupwayi *et al.*, 2011, Roberts *et al.*, 2011, Marschner *et al.*, 2003, Ogilvie *et al.*, 2008).

N and C cycling in soils are interlinked and plays a critical role in maintaining soil nutrients which are directly related to crop productivity (Gasser & Lake, 1982, Gougoulias *et al.*, 2014). Thus, it is imperative to examine the response of C cycling processes to the legacy effects of extreme weather events and N supply. Soil microbial respiration, a proxy to soil organic C decomposition, in response to altered precipitation is highly variable and dependent on the ecosystem (Borken *et al.*, 2006, Cleveland *et al.*, 2010, Van Straaten *et al.*, 2010). This process is an important flux in the soil C cycle and linked to soil organic C pools (Gougoulias *et al.*, 2014). Additionally, the underlying mechanism of soil microbial respiration in response to N fertilizer addition remains relatively unclear (Ramirez *et al.*, 2010).

Based on results from Chapter 4 of the thesis, this chapter examined the response of the entire soil bacterial community and microbial respiration to legacy effects of extreme weather events and different rates of N fertilizer addition. I hypothesized that soil total bacterial communities and microbial respiration will respond to the legacy effects of prolonged-drought, waterlogging and N addition due to altered soil physicochemical properties.

5.2 Materials and Methods

5.2.1 Soil bacterial community analysis

The experimental design and soil sampling procedures are described in detail in Chapter 4 (section 4.2.1 & 4.2.2).

Total soil genomic DNA was extracted using the MoBio PowerSoil DNA Isolation kit (MoBio Laboratories, Carlsbad, CA, USA) according to manufacturer's instruction. The detailed procedure is outlined in Chapter 2 (section 2.2.7.1). Extracted DNA was kept at -20^{o}C for further manipulations.

Bacterial abundance was quantified by quantitative PCR (qPCR) of the 16S rRNA gene using the primer Eub338f/Eub518r (ACTCCTACGGGAGGCAGCAG/ATTACCGCGGCTGCTGG)

(Fierer *et al.*, 2005). Each sample was quantified in a 10 µl reaction including 5 µl GoTaq® qPCR Master Mix (2X), 20µM each primer, 0.1 µl CXR reference dye and 10 ng of template. The PCR thermal cycling conditions were as follows: an initial cycle of 95°C for 15 min, 40 cycles of 95°C for 1min, 53°C for 30 s, to 72°C for 1 min, and 1 cycle of 95°C for 15 s, 60°C for 15 s, to 95°C for 15 s (Fierer *et al*., 2005).

Standard plasmids for 16S rRNA were constructed by cloning PCR products into the pGEM-T easy vector (Promega, Madison, USA). Calibration curves were generated using a 10-fold serial dilution of plasmids. Melt-curve analyses (from 65 to 95°C) were conducted following each assay to verify the specificity of the amplification products. PCR efficiency values for the abundance of 16S rRNA was in the range of 95-101%.

The soil bacterial diversity and community composition were examined by 16S amplicon sequencing. In particular, the region V3-V4 of 16S rRNA gene was sequenced on the MiSeq platform (Illumina, San Diego, CA, USA) by the Next Generation Sequencing Service at Western Sydney University, NSW. The primers for PCR amplification to produce paired-ends reads of 301 bp were 341F/805R (CCTACGGGNGGCWGCAG/GACTA CHVGGGTATCTAATCC) (Herlemann *et al.*, 2011). The sequence analysis was conducted by using the Quantitative Insights into Microbial Ecology (QIIME) 1.7.0 (Caporaso *et al.*, 2010b). The 'SeqPrep' method was used to join paired ends (https://github.com/jstjohn/SeqPrep). Barcodes, linkers and reverse primer sequences of the raw sequence reads with quality score ≥ Q20 were removed. Chimeric sequences were filtered out using USEARCH (Edgar, 2010, Edgar *et al.*, 2011) against the Greengenes database (DeSantis *et al.*, 2006). Sequences were then clustered into operational taxonomic units (OTUs) at a 97% identity threshold using uclust (Edgar, 2010). Representative sequences from individual OTUs were then aligned using PyNAST (Caporaso *et al.*, 2010a) and phylogeny was assigned using ribosomal database project (RDP) Classifier (Wang *et al.*, 2007) based on the Greengenes database (DeSantis *et al*., 2006). Each sample was resampled according to the minimum sequence numbers before the downstream analyses. Bacterial diversity was then characterized by calculating

Shannon index. Principal coordinates analysis (PCOs) for Bray-Curtis dissimilarity matrices was applied to visualize shifts in the microbial community compositions based on the 97% OTU level across different treatments (Caporaso *et al.*, 2010b).

5.2.2 Soil microbial respiration

Community respiration was examined by MicroResp™ (Campbell *et al.*, 2003). In particular, two 96-deep well micro-titre plates for soil and C substrate and CO_2 detection plate were used in this experiment. The detection plate containing a gel-based bicarbonate buffer with cresol red indicator dye which responds to changes in pH due to the gel absorbance of evolved CO_2 from carbon substrate utilization. Each soil sample (0.4 g) was transferred into the 96-deep well plates which were then incubated for two days at field moisture in the dark before carrying out the assay. Two substrates including glucose and lignin (Sigma Aldrich, Australia) with concentration of 1 mg ml^{-1} per well were used in this study to examine C mineralization capacity (Colombo *et al.*, 2016). Basal respiration was also determined by using sterile deionized water. Both basal and substrate-induced respiration were measured in four replicates for each sample.

Prior to assembling the 96-deep well plates, the optical density of the detection plate was measured by the Microplate reader (CALRIOstar®, BMG LabTech, Mornington, Australia) at the wave length of 570 nm. After that, the assemblage of the detection plate and the 96-deep well plate was incubated at 25°C for 6 hours. The change in optical density of the CO_2-detection plate was measured again after 6 hours of incubation. The rate of CO_2 respiration per gram of dry soil was calculated using the following formula as described in MicroResp™ manual (Macaulay Scientific Consulting, UK).

$$CO_2\ g^{-1}\text{dry soil} = \frac{\left(\%\frac{CO_2}{100}\right) \times \text{headspace volume} \times \left(\frac{44}{22.4}\right) \times \left(\frac{12}{44}\right) \times \left(\frac{273}{273 + 25℃}\right)}{\frac{\text{soil fresh weight x (soil \% dry weight)}}{\text{incubation time}}}$$

5.2.3 Statistical analysis

One-way ANOVA was used to examine the legacy effects of extreme weather events on the soil bacterial diversity and abundance, and microbial respiration at pre-planting.

Two-way ANOVA with Tukey's HSD was applied to test the effects of N-addition, legacy effects of extreme weather events, and their interaction on the soil bacterial diversity and abundance, and microbial respiration. To determine the effects of cotton growth stages, N-addition, extreme weather legacy effects and their interactions on the abundance of 16S rRNA gene and microbial respiration, two-way repeated measures ANOVA was carried out.

The copy number of 16S rRNA gene was log-transformed prior to statistical analysis to satisfy normality assumptions. Spearman's rank correlation analysis was applied to test the relationship between soil physicochemical properties and soil microbial respiration; the abundance, diversity and composition of total bacteria and microbial respiration rates. Stepwise regression analysis was conducted to examine the predictors of changes in the abundance, diversity, and composition of total bacteria.

PCO analysis was used to examine the shifts in bacterial composition based on Bray-Curtis matrices, and PERMANOVA was conducted to test the significance of Bray-Curtis dissimilarity.

$P<0.05$ was considered to be statistically significant. All tests were manipulated in SPSS 22 (IBM, Armonk, USA) and Primer v6 (Primer-E Ltd, Plymouth, UK).

5.3 Results

5.3.1 The abundance of soil bacterial community

The copy number of 16S rRNA for control and Post-WL soils prior to planting soil were 2.48×10^9 and 2.93×10^9/g dry soil, respectively. No significant difference in total bacterial abundance was observed for control and Post-WL soils ($P=0.087$). The

abundance of total bacteria of Post-PD soils was 6.7-fold lower than that of control. One-way ANOVA showed significant difference in 16S rRNA gene abundance between control and Post-PD soils (P=0.01).

The 16S rRNA gene abundance was also determined for samples collected at the early squaring and flowering stages, as well as at harvest. Total bacterial abundance of control and Post-WL soils varied from 4.13 x 10^9 to 6.62 x 10^9, and from 4.29 x 10^9 to 6.88 x 10^9 copies/g dry soil, respectively whereas that of Post-PD ranged from 1.31 x 10^9 to 4.22 x 10^9 copies/g dry soil across all treatments and cotton developmental stages. At each developmental stage, with the same rate of N-addition, the total bacterial abundance of control and Post-WL soils were not significantly different. In contrast, the 16S rRNA gene abundance of Post-PD soils was significantly lower than that of control soils. Additionally, only significant differences in the 16S rRNA gene abundance were detected between the rates of 300 kg N/ha and 0 kg N/ha for Post-WL and control soils at the early squaring stage. For Post-PD soils, there were significant differences between 0, 100 kg N/ha and 300 kg N/ha, and this trend was maintained for all cotton developmental stages. At the early flowering stage and harvest, the 16S rRNA gene abundance of Post-WL soils was significantly different between the rates of 0 and 300 kg N/ha whereas that of Post-PD soils was significantly different between the rates of 0, 200 and 300 kg N/ha (**Figure 5.1**).

Two-way repeated measures ANOVA indicated the significant effects of cotton growth stage and N addition on 16S rRNA gene abundance of Post-WL and Post-PD soils (P<0.001, P=0.007 for Post-WL; and P<0.001, P=0.023 for Post-PD). Prolonged-drought placed a legacy effect on this variable (P<0.001). No interaction between factors was observed.

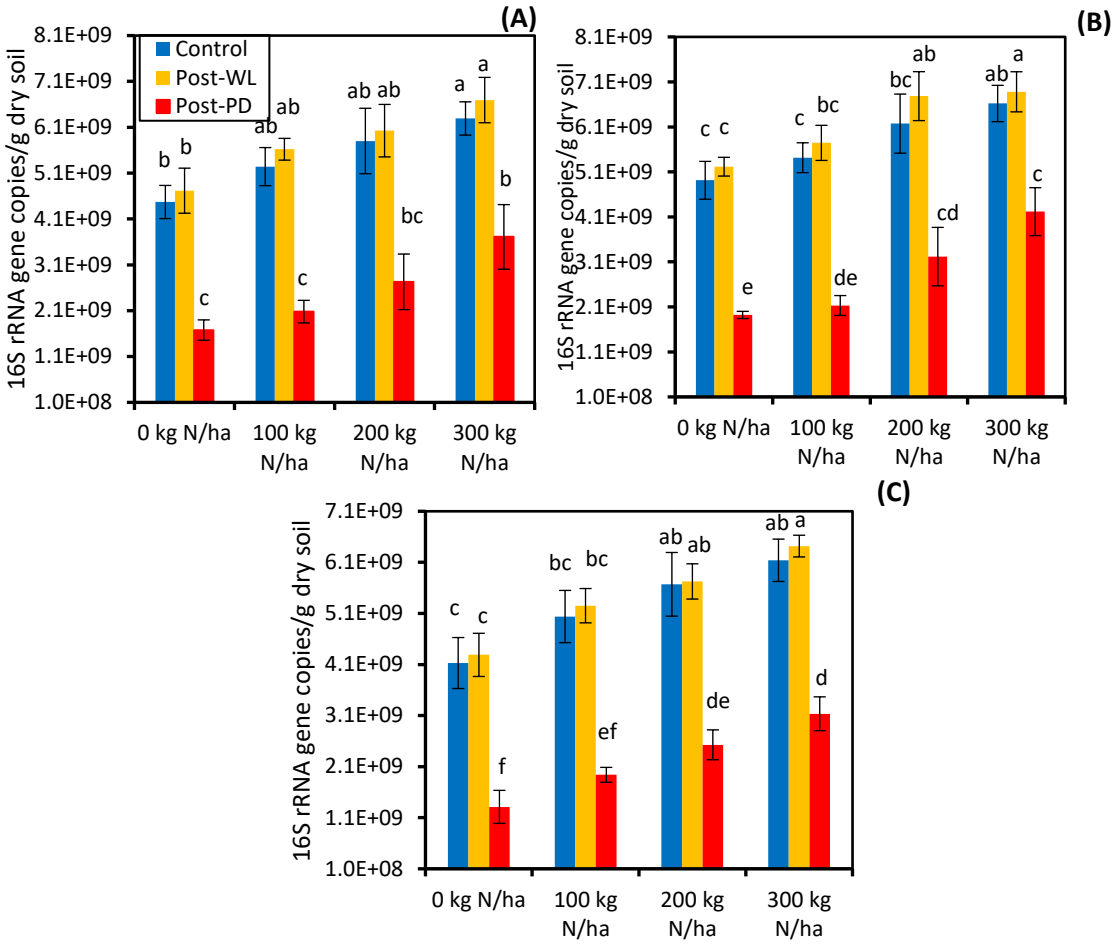

Figure 5.1 The abundance of total bacteria across all soil treatments and N fertilizer rates at (**A**) the early squaring, (**B**) the early flowering, and (**C**) harvest. Values represent mean ± SE (n=4) of each soil water treatment at each N level. Different letters indicate significant differences between control and Post-WL soils, and control and Post-PD soils at each N fertilizer level and among N fertilizer levels. Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought.

5.3.2 The diversity and community composition of soil bacteria

Illumina Miseq across all 72 samples yielded 24,036,397 high-quality bacterial 16S rRNA gene sequences and the minimum number of sequences for individual samples was 62,855. Prior to planting, the Shannon index of control and Post-WL soils was not significantly different (*P*=0.068) whereas that of Post-PD soils was significantly lower

than control soils (P=0.001) (**Figure 5.2A**). At the early flowering stage, there were upward trends of the Shannon index across all soil water treatments and N-addition. Two-way ANOVA showed significant effects of previous prolonged-drought (P<0.001). Similarly, N supply also significantly affected Shannon index (P<0.001); however, no significantly interactive effects of drought legacy and N supply were observed (P=0.943). Regarding Post-WL soils, no legacy effects were established (P=0.192). N-addition significantly affected Shannon index of Post-WL soils (P=0.002). In contrast, no significant interactive effects of N supply and waterlogging legacy were observed (P=0.949) (**Figure 5.2B**).

PCO analysis of the bacterial community data showed clear separation between control and Post-PD at pre-planting while control and Post-WL samples clustered together (**Figure 5.3A**). However, PERMANOVA test indicated that the bacterial community composition of control and Post-WL soils was significantly different (F=9.6615, P=0.002) at pre-planting. Similarly, the bacterial community composition of control soils significantly differed from that of Post-PD soils (F=41.099, P=0.001). At the early flowering stage, similar trends were observed. Particularly, there was clear separation of the bacterial community composition between control and Post-PD soils while the distinction between control and Post-WL was less apparent (**Figure 5.3B**). PERMANOVA showed significant effects of previous waterlogging and prolonged-drought on the bacterial community composition (F=19.181, P=0.001; and F=66.299, P=0.001, respectively). N supply significantly affected the bacterial community composition of Post-WL soils (F=4.6087, P=0.001) whereas no significant effects of N addition on the bacterial composition of Post-PD soils (F=1.4908, P=0.074). No significant interactive effects of N supply and previous waterlogging on the bacterial community composition were observed (F=1.1107, P=0.311). In contrast, the interaction of previous prolonged-drought and N addition significantly affected the bacterial community composition (F=4.776, P=0.001).

The bacterial community across all soil water treatments and N-addition rates comprised of 11 main phyla, in which *Proteobacteria* (13.29-28.37%), *Acidobacteria* (7.47-18.2%), *Actinobacteria* (7.32-27.9%), *Bacteroidetes* (4.65-19.97%), and *Plantomycetes* (3.69-17.72%) were the dominant phyla (**Figure 5.4**). At pre-planting, compared with the control soils, the relative abundance of *Proteobacteria*, *Bacteroidetes*, and *Planctomycetes* were significantly lower in Post-PD soils (P=0.012, P=0.023, P=0.011, respectively) whereas that of *Acidobacteria*, *Actinobacteria* and *Chloroflexi* were not significantly different (P=0.13, P=0.092 and P=0.42, respectively). In contrast, the relative abundance of all phyla between control and Post-WL soils were not significantly different (P>0.05). At the early flowering stage, no significant differences in the relative abundance of all phyla between control and Post-WL soils at the same rate of N supply was observed. Although N supply increased the relative abundance of *Proteobacteria*, *Planctomycetes* and *Bacteroidetes*, these phyla were significantly lower in Post-PD soils compared to control soils at each rate of N supply. The relative abundance of two dominant phyla *Acidobacteria* and *Actinobacteria* decreased with the increasing N fertilizer addition rates (**Figure 5.4**).

Stepwise regression analysis was applied to reveal the mechanisms which mostly influenced soil bacterial communities. The bacterial diversity and composition were significantly related to soil NH_4^+ content and total N, respectively (P<0.001 for the bacterial diversity and P=0.002 for the bacterial community composition). 16S rRNA gene abundance was significantly related to soil NO_3^- content (P<0.001) (**Table 5.1**).

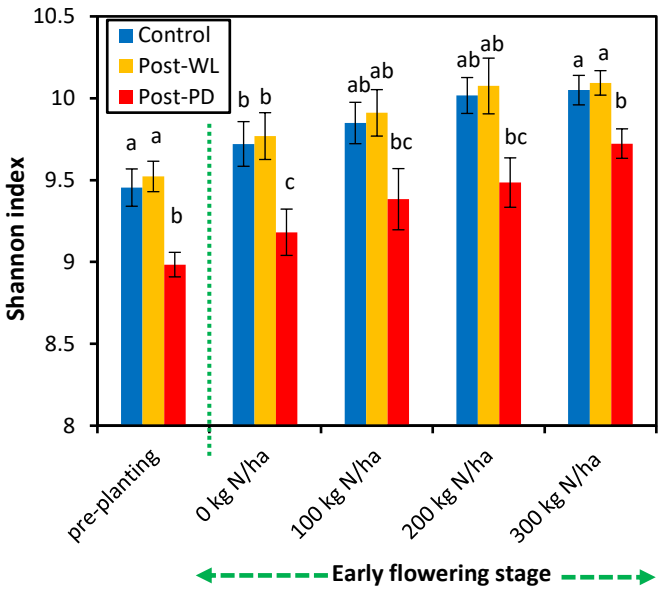

Figure 5.2 Shannon index of soil total bacteria community at pre-planting and at the early flowering stage across all soil water treatments and N-addition rates. Values represent mean ± SE (n=4) of each soil water treatment at each N level. Different letters indicate significant differences between control and Post-WL soils, and control and Post-PD soils at pre-planting and at the early flowering stage. Post-WL=Post-waterlogging, Post-PD= Post-prolonged drought.

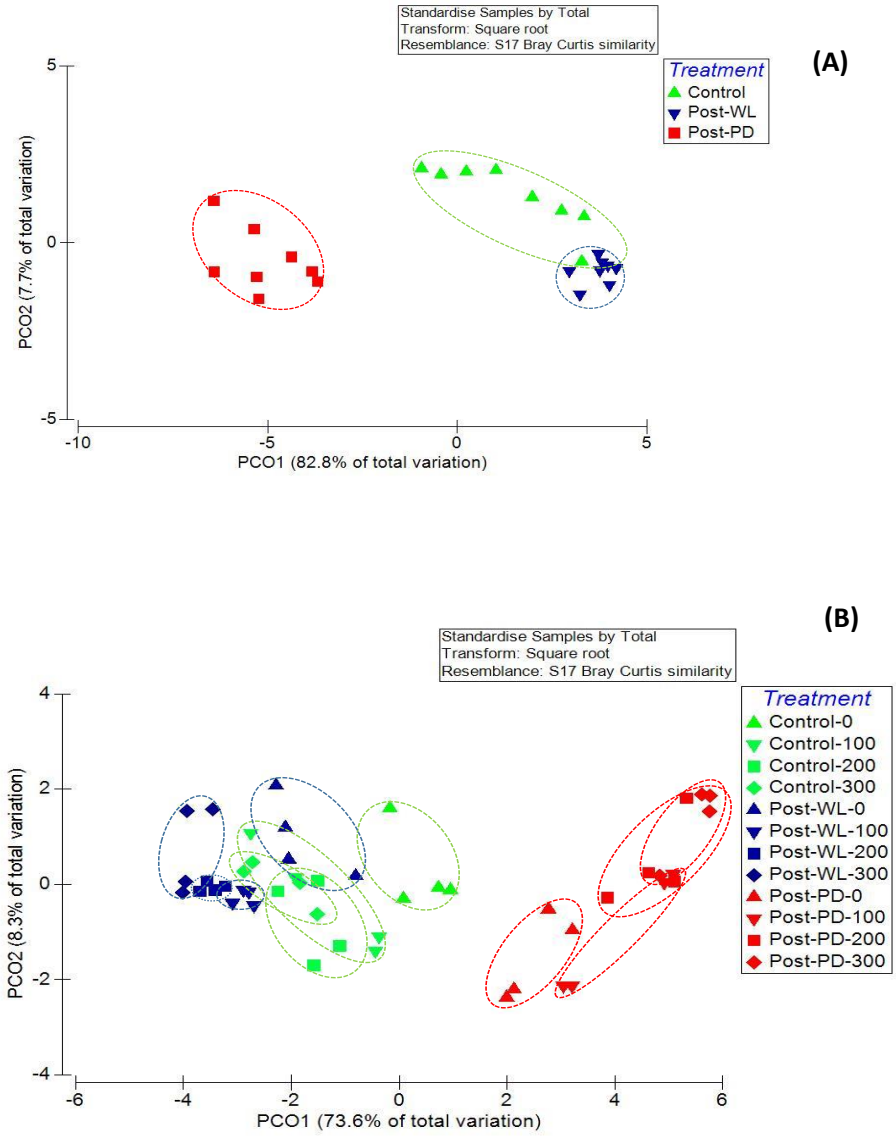

Figure 5.3 Principal coordinates analysis (PCO) were derived from the Bray-Curtis dissimilarity matrices, based on the 97% OTU level of bacterial community compositions across all soil water treatments and N-addition at (**A**) pre-planting and (**B**) the early flowering stage. Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought. OTU = Operational Taxonomic Unit.

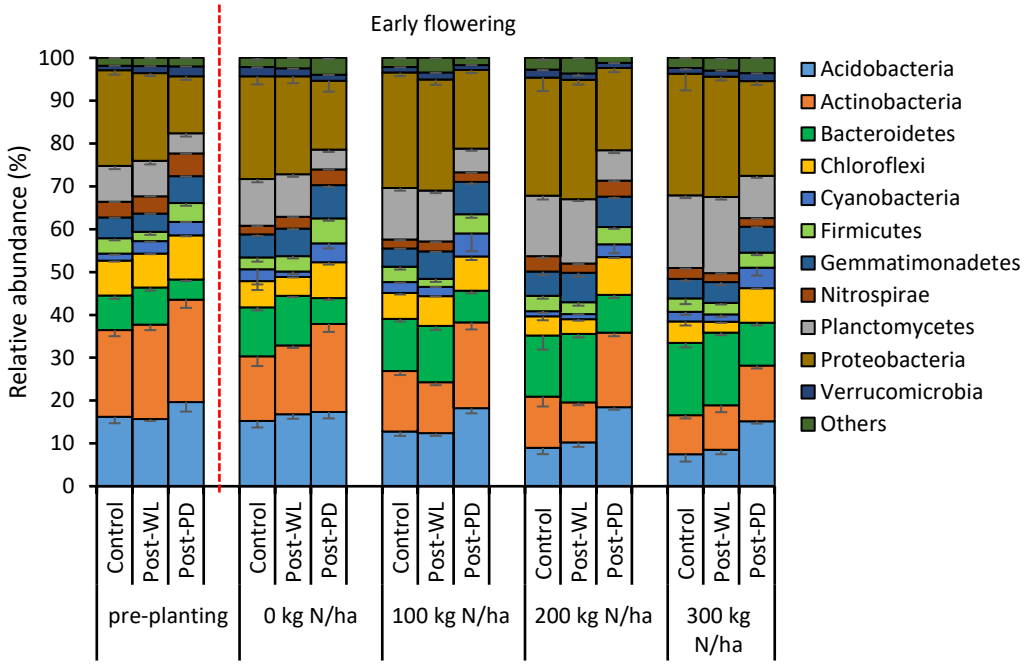

Figure 5.4 Changes in the bacterial community compositions at the phylum level across all soil water treatments and N-addition at pre-planting and the early flowering stage. Post-WL=Post-waterlogging, Post-PD=Post-prolonged drought.

Table 5.1 Variables responsible for changes in the soil bacterial abundance, diversity, and compositions. Significant differences at $P<0.05$ are in bold. R^2 = The coefficient of determination, F = The F-value, P= The P-value. NH_4^+ = Ammonium, NO_3^- = Nitrate, N = Nitrogen.

Index	Result	R^2	F	P
Diversity	y= 8.939 + 1.072 (soil NH_4^+ content)	0.485	43.352	**<0.001**
Abundance	y= 518.363 - 6.79 (soil NO_3^- content)	0.453	58.309	**<0.001**
Composition	y= -4.217 + 5.559 (soil total N content)	0.289	10.712	**0.002**

5.3.2 Soil microbial respiration

Before planting, basal, glucose-induced and lignin-induced respiration varied from 0.61 to 0.76, 0.79 to 0.96 and 0.87 to 1.15 μg CO_2-C/g/h, respectively across all soil water treatments. Microbial respiration rates of control and Post-WL soils were similar (*P*=0.45, *P*=0.13 and *P*=0.098 for basal, glucose and lignin-induced respiration, respectively) whereas that of Post-PD was significantly lower (*P*=0.005, *P*=0.02 and *P*=0.009 for basal, glucose and lignin-induced respiration, respectively) (**Figure 5.5**).

Soil microbial respiration was measured when plants were at the early flowering stage. Basal respiration rate varied from 0.687 to 0.876 μg CO_2-C/g/h for control samples. In terms of Post-WL and Post-PD soils, basal respiration rate varied from 0.676 to 0.856, and 0.4512 to 0.675 μg CO_2-C/g/h, respectively. Glucose-induced respiration rates of control and Post-WL soils ranged from 0.874 to 1.243, and 0.855 to 1.221 μg CO_2-C/g/h whereas it varied from 0.632 to 0.91 μg CO_2-C/g/h for control soils. Lignin-induced microbial respiration rates varied from 0.981 to 1.481, 0.7 to 1.38 and 0.7 to 1.054 μg CO_2-C/g/h for control, Post-WL and Post-PD, respectively. No significant differences in respiration rates between control and Post-WL soils at the same rate of N addition were observed. Additionally, microbial respiration decreases with the increase in N supply. Within the same water treatment, the significant differences were only observed between soils with the rate of 0 kg N/ha and 300 kg N/ha (**Figure 5.5**).

Spearman's rank correlation analysis indicated that microbial respiration rates were significantly correlated with soil inorganic N (**Table 5.2**).

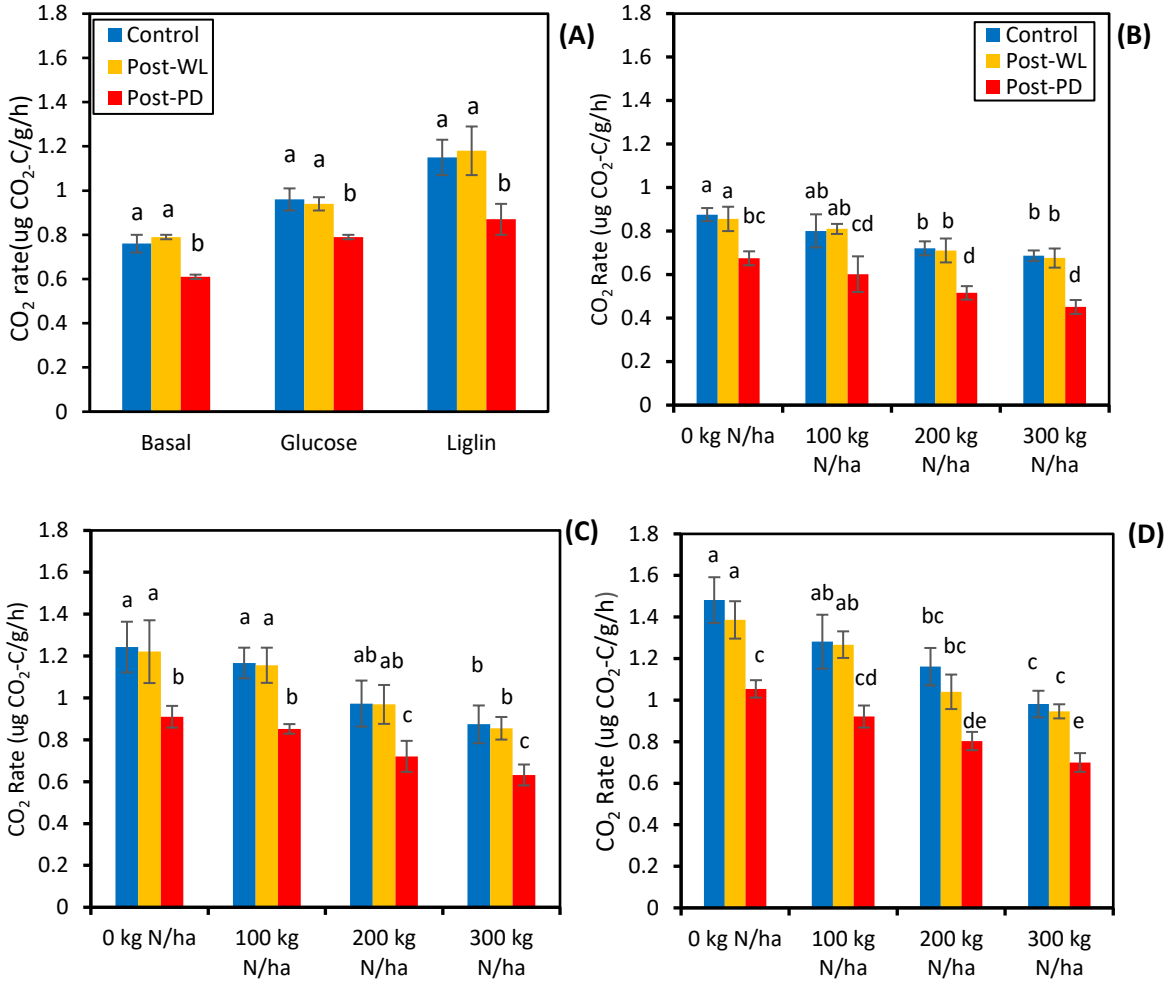

Figure 5.5 Microbial respiration at (**A**) pre-planting and the early flowering stage across all soil treatments and different nitrogen fertilizer rates: (**B**) Basal respiration, (**C**) Glucose-induced respiration, and (**D**) Lignin-induced respiration. Values represent mean ± SE (n=4) of each soil water treatment at each N level. Different letters indicate significant differences between treatments at pre-planting and the early flowering stage. Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought.

Table 5.2 Correlation coefficients between soil physicochemical properties and microbial respiration rates. Significant differences at $P<0.05$ are in bold. NH_4^+ = Ammonium, NO_3^-= Nitrate, Total N = Total nitrogen, Total C = Total carbon.

Variables	Soil moisture	pH	NH_4^+	NO_3^-	Total N	Total C
Basal	0.105	0.012	**-0.257***	**-0.278***	-0.156	0.013
Glucose	0.037	0.302	**-0.294***	**-0.361***	-0.186	0.054
Lignin	0.059	0.255	**-0.294***	**-0.391***	-0.21	0.101

5.3.3 Relationship between soil bacterial community and microbial respiration rates

Relationships between soil bacterial abundance, diversity, composition, and microbial respiration (basal, glucose and lignin-induced respirations) for each soil water treatment and N fertilizer rate were examined by using Spearman's rank correlation analysis. At pre-planting, no significant correlations between bacterial abundance and basal respiration and glucose-induced respiration for control, Post-WL and Post-PD soils were observed (**Table 5.3**). In contrast, there were statistically significant correlation between bacterial abundance and lignin-induced respiration for all soil water treatments (r_S= 0.415, P=0.012 for control; r_S=0.392, P=0.03 for Post-WL; r_S=0.426, P=0.011 for Post-PD, **Table 5.3**).

There were significant correlations between bacterial diversity and basal respirations for all soil water treatments (r_S=0.405, P=0.01 for control; r_S=0.410, P=0.031 for Post-WL; r_S=0.424, P=0.015 for Post-PD, **Table 5.3**). Similar trend was observed for relationship between bacterial diversity and glucose-induced respiration (r_S=0.433, P=0.012 for control; r_S=0.450, P=0.011 for Post-WL; r_S=0.462, P=0.022 for Post-PD, **Table 5.3**). No

significant correlations between bacterial diversity and lignin-induced respiration were found (r_S=0.306, *P*=0.091 for control; r_S=0.303, *P*=0.121 for Post-WL; r_S= 0.317, *P*=0.083 for Post-PD).

Bacterial composition was significantly correlated with basal respiration for control and Post-WL soils (r_S=0.402, *P*=0.015; r_S=0.412, *P*=0.011 for control and Post-WL, respectively. No significant correlations between bacterial composition and glucose and lignin-induced respiration were observed (**Table 5.3**).

The relationship between total bacterial community and microbial respiration was also examined for each soil water treatment with different N fertilizer rates. Significant correlations between bacterial abundance and lignin-induced respiration were observed for all soil water treatments only at N fertilizer rate of 0 kg N/ha (r_S=0.407, *P*=0.014 for control; r_S=0.411, *P*=0.021 for Post-WL; r_S=0.395, *P*=0.011 for Post-PD, **Table 5.4**).

Similarly, there were significant correlations between bacterial diversity and basal and glucose-induced respirations for all soil water treatments without N fertilizer addition. Bacterial diversity was significantly correlated with glucose-induced respiration for all soil water treatments with N fertilizer rate of 100 kg N/ha (r_S=0.45, *P*=0.024 for control; r_S=0.411, *P*=0.013 for Post-WL; r_S=0.401, *P*=0.017 for Post-PD, **Table 5.4**). At N fertilizer rate of 100 and 200 kg N/ha, there were significant correlations between bacterial diversity and basal respiration for control and Post-PD soils (r_S=0.381, *P*=0.022 and r_S= 0.397, *P*=0.014 for control and Post-PD soils, respectively at 100 kg N/ha; r_S=0.385, *P*= 0.014, r_S=0.387, *P*=0.031 for control and Post-PD soils, respectively at 200 kg N/ha). At rate of 300 kg N/ha, bacterial diversity was significantly correlated with basal respiration for all soil water treatments (r_S=0.375, *P*=0.025 for control; r_S=0.371, *P*=0.03 for Post-WL; r_S=0.372, *P*=0.027 for Post-PD, **Table 5.4**).

There was a significant correlation between bacterial community composition and basal respiration for control soils at 0 kg N/ha (r_S=0.371, *P*=0.021). No significant correlations between bacterial community composition and basal respiration for Post-WL and Post-

PD soils at 0 kg N/ha were observed (**Table 5.4).** Glucose-induced respiration was also significantly correlated with bacterial community composition for all soil water treatments without N addition (r_S=0.471, *P*=0.013; r_S=0.462, *P*=0.011, r_S=0.437, *P*=0.022 for control, Post-WL, Post-PD, respectively). Significant correlations between bacterial community composition and lignin-induced respiration for all soil water treatments were observed at 0 kg N/ha (r_S=0.373, *P*=0.031; r_S=0.378, *P*=0.025; r_S=0.317, *P*=0.027 for control, Post-WL and Post-PD soils, respectively, **Table 5.4**). No significant correlations between bacterial community composition and basal, glucose, and lignin-induced respirations for all soil water treatments at 100, 200 and 300 kg N/ha were observed, **Table 5.4**)

Overall, there were slight differences in the relationship between bacterial community (bacterial abundance, diversity, and composition) and microbial respiration (basal, glucose and lignin-induced respirations) among soil water treatments. In other words, waterlogging and prolonged-drought events prior to planting did not affect the relationships between soil bacterial community and microbial respiration. However, N fertilizer addition weakened these relationships.

Table 5.3 Correlation coefficients between soil bacterial abundance, diversity, composition, and microbial respiration (basal, glucose and lignin-induced respiration) at pre-planting. Significant differences at *P*<0.05 (*) are in bold. Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought

	Control	**Post-WL**	**Post-PD**	**Control**	**Post-WL**	**Post-PD**	**Control**	**Post-WL**	**Post-PD**
Variables	**Basal respiration**			**Glucose-induced respiration**			**Lignin-induced respiration**		
Abundance	0.306	0.284	0.169	0.345	0.313	0.19	**0.415***	**0.392***	**0.426***
Diversity	**0.405***	**0.410***	**0.424***	**0.433***	**0.450***	**0.462***	0.306	0.303	0.317
Composition	**0.402***	**0.412***	0.381	0.326	0.336	0.313	0.303	0.306	0.317

Table 5.4 Correlation coefficients between soil bacterial abundance and microbial respiration (basal, glucose and lignin-induced respiration) at each N fertilizer rate during growing season; and soil bacterial diversity, composition, and microbial respiration (basal, glucose and lignin-induced respiration) at each N fertilizer rate at the early flowering. Significant differences at $P<0.01$(**) and $P<0.05$ (*) are in bold. Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought.

	0 kg N/ha			**100 kg N/ha**			**200 kg N/ha**			**300 kg N/ha**		
	Control	**Post-WL**	**Post-PD**	**Control**	**Post-WL**	**Post-PD**	**Control**	**Post-WL**	**Post-PD**	**Control**	**Post-WL**	**Post-PD**
Variables	**Basal respiration**											
Abundance	0.315	0.320	0.295	0.311	0.306	0.301	0.318	0.307	0.312	0.264	0.262	0.258
Diversity	**0.461***	**0.442***	**0.415***	**0.381***	**0.397***	0.332	**0.385***	**0.387***	0.317	**0.375***	**0.371***	**0.372***
Composition	**0.371***	0.333	0.327	0.312	0.311	0.317	0.301	0.296	0.292	0.284	0.303	0.266
	Glucose-induced respiration											
Abundance	0.328	0.335	0.275	0.309	0.311	0.284	0.267	0.266	0.247	0.271	0.264	0.187
Diversity	**0.421***	**0.418***	**0.423***	**0.405***	**0.411***	**0.401***	0.386	0.397	**0.403***	0.275	0.302	0.294
Composition	**0.471****	**0.462***	**0.437***	0.365	0.342	0.348	0.303	0.311	0.306	0.276	0.285	0.274
	Lignin-induced respiration											
Abundance	**0.407***	**0.411***	**0.395***	0.354	0.342	0.329	0.333	0.301	0.312	0.301	0.297	0.315
Diversity	0.311	0.309	0.307	0.275	0.301	0.254	0.295	0.284	0.291	0.253	0.248	0.254
Composition	**0.373***	**0.378***	**0.371***	0.284	0.282	0.262	0.302	0.267	0.244	0.238	0.221	0.224

5.4 Discussion

Prolonged-drought event prior to planting established negative legacy effects on soil functional microbial communities (AOB and N_2O-reducing bacteria) and their functions, whereas waterlogging before sowing had minor legacy effects (Chapter 4). As soil C and N cycling are interlinked, it is crucial to identify whether extreme weather events establish legacy effects on the C cycling. Thus, in this chapter, total soil bacterial community and microbial respiration after exposure to waterlogging and prolonged-drought were examined. Similar to the findings in Chapter 4, strong legacy effects of prolonged-drought prior to planting on the soil bacterial community and microbial respiration were observed. In contrast, previous waterlogging did not generate legacy effects on these measurements. It suggests that the soil total bacterial community and microbial respiration might be resistant to waterlogging or might be able to recover completely. External N supply has been shown to improve soil fertility, and hence four different rates of N fertilizer were used with purpose of fertilizing soils after exposure to extreme weather events. The supply of up to 300 kg N/ha could not counteract the drought legacy effects on soil bacterial communities. I also found that different phyla responded differently to legacy effects of prolonged-drought and N fertilizer addition, and the N addition inhibited soil microbial respiration rates.

Prolonged-drought and re-wetting stresses in this study resulted in microbial mortality. At pre-planting, the relative abundance of three dominant phyla, particularly *Actinobacteria*, *Acidobacteria* and *Chloroflexi*, were resistant to prolonged-drought. The phylum *Actinobacteria* is Gram-positive bacteria capable of forming spores and resisting to drought conditions (Singh *et al.*, 2007). In contrast, the relative abundance of *Proteobacteria*, *Bacteroidetes* and *Planctomycetes* were negatively affected by prolonged-drought. *Proteobacteria and Bacteroidetes* are Gram-negative and characterized to be highly vulnerable to environmental disturbances and water limitation stress (Uhlířová *et al.*, 2005, Schimel *et al.*, 2007). The phylum *Planctomycetes* is also Gram-negative and slow-growing bacteria, and hence may take a long time to

recover after environmental stresses (Buckley *et al.*, 2006). My results showed that N fertilizer supply increased gradually the relative abundance of these phyla across all soil water treatments. This finding is supported by previous observations of the resilience in microbial communities to environmental disturbance when living conditions were improved (Singh *et al.*, 2007, Lu *et al.*, 2006).

Stepwise regression analysis indicated soil NH_4^+, NO_3^- and total N were responsible for changes in total bacterial diversity, abundance, and composition, respectively. Thus, this supports my hypothesis that changes in soil physicochemical properties due to legacy effects of extreme weather events and N addition will lead to shifts in soil bacterial communities.

Soil microbial respiration was observed to decrease with increases in N fertilizer addition in my study. This is in agreement with a number of previous studies showing the inhibition of N supply on soil microbial respiration (Thirukkumaran & Parkinson, 2000, Bowden *et al.*, 2004, Craine *et al.*, 2007, Ramirez *et al.*, 2010). My findings of significantly negative correlations between soil NH_4^+, NO_3^- contents and microbial respiration supports the hypothesis that soil microbial respiration will respond to legacy effects of drought x N fertilizer addition due to changes in soil physicochemical properties. This agrees with the study by Ramirez *et al*., (2010) who observed negative direct effects of increases in N availability due to N fertilizer supply on soil microbial respiration rates.

Interestingly, N supply increased the abundance and diversity of soil total bacteria, but decreased microbial respiration rates in my study. The decreased microbial respiration due to N fertilizer addition could be attributed to (i) inhibited C-degraded enzyme activities (Berg & Matzner, 1997, Gallo *et al.*, 2004, Sinsabaugh *et al.*, 2005, Waldrop & Zak, 2006), or (ii) shifts in the microbial community composition (Fontaine *et al.*, 2003, Fierer *et al.*, 2007, Fierer *et al.*, 2012). In this study, no significant correlations between soil bacterial community composition and microbial respiration were observed for control, Post-WL and Post-PD soils upon N fertilizer application although there were changes in bacterial community composition due to N addition. Thus, my findings

support the premise that N supply inhibits enzymes involving in decomposing recalcitrant C, thereby reducing microbial respiration rates (Gallo *et al*., 2004).

In Chapter 4, N fertilizer addition increased N availability in soils via direct increases in NH_4^+ and NO_3^- in soils and indirectly stimulated nitrification. In this chapter, I found inhibited soil microbial respiration upon N supply, and thus reducing C release from soils. Therefore, increases in crop productivity observed in Chapter 4 with increased N fertilizer rates could be explained by both enhanced soil N availability and C sequestration in my study.

5.5 Conclusions

The prolonged-drought period established legacy effects on the soil bacterial communities and microbial respiration whereas only marginal or no legacy effects of waterlogging events on these variables were observed. Different groups of bacteria responded differently to these legacy effects; three phyla (*Proteobacteria*, *Bacteroidetes* and *Phanctomycetes*) were significantly decreased. N fertilizer supply could not completely diminish these negative legacy effects on soil bacterial communities, suggesting that they will take a long time to recover. N supply decreased soil microbial respiration rates, thereby limiting C loss from soils. The dominant phyla *Acidobacteria* and *Actinobacteria* decreased with increased N fertilizer addition rates. A greater understanding of soil bacterial communities and respiration in response to legacy effects, due to extreme weather events, will help to develop adaptation and management strategies to sustain soil functions and fertility, and thus maintain crop yields.

CHAPTER 6 GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

Conclusions

In this thesis, I provided novel evidence for the role of soils in modulating crop productivity responses to climate change and extreme weather events by conducting laboratory and field-based experiments. This work is also the first to investigate legacy effects of extreme weather events and the interaction of these legacy effects and N fertilizer supply on soil biotic and abiotic properties, and subsequent consequences for crop yields. Throughout this project, I mainly focused on soil N cycling processes: nitrification (**Chapters 2, 3 and 4**), N mineralization and denitrification (**Chapter 4**) and the functional microbial communities, particularly nitrifiers (**Chapters 2, 3 and 4**) and denitrifiers (**Chapter 4**). Additionally, the soil total bacterial community and C cycling (microbial respiration) (**Chapter 5**) in response to legacy effects of extreme weather events and N fertilizer addition were examined to elucidate linkage between C and N cycles. Following are the most important findings in this thesis (**Figures 6.1; 6.2; and 6.3**):

- Waterlogging events at the early and late flowering stage of cotton plants significantly decreased the rate of soil nitrification through shifts in ammonia-oxidizing communities due to unfavorable conditions including high soil water content and pH. The hypoxia or even anoxia upon waterlogging might also facilitate N loss via leaching and denitrification. Consequently, soil NO_3^- content significantly decreased upon waterlogging, resulting in the depletion of soil nutrient availability which might negatively influence crop productivity (**Chapter 2**).
- Elevated temperature (+1.1^{o}C) did not significantly affect soil nitrification rate and AOB. Only AOA responded to elevated temperature, however, the shifts in the AOA community did not lead to changes in nitrification activity. It could be

due to alterations in the dormant fraction of AOA, and thus the overall nitrification kinetics were not affected. The response of AOA to elevated temperature was also dependent on crop growth stage (**Chapter 3).**

- Elevated CO_2 (550ppm) in combination with elevated temperature (+1.1^oC) significantly impacted ammonia-oxidizing communities by influencing soil and plant properties, resulting in an increase in nitrification rate which was related to soil nutrient availability and crop productivity. However, the response of nitrification to combined elevated CO_2 and temperature was dependent on cotton growth stage since changes in the rate of nitrification and ammonia-oxidizing community were observed only when cotton plants reached the early flowering stage (**Chapter 3**).
- Prolonged-drought prior to planting established strong legacy effects on soil and crop productivity. Although N fertilizer addition enhanced soil N availability, N addition up to 300 kg N/ha could not counteract the drought legacy effects on crop yields (**Chapter 4**).
- Waterlogging prior to planting had weak legacy effects on soil and crop productivity. However, waterlogging events prior to planting placed a legacy effect on soil NO_3^- and *nosZ*-containing community. The legacy effects of waterlogging on soil NO_3^- level at pre-sowing was compensated by N addition and hence crop productivity was maintained. However, if any further waterlogging occurs, soil N availability may be depleted due to higher abundance of denitrifying community and consequently can potentially influence the crop productivity (**Chapter 4**).
- Prolonged-drought prior to planting also had strong legacy effects on the soil total bacterial community and microbial respiration, suggesting that C cycling may respond to drought legacy effects as N cycling did. N fertilizer supply stimulated soil bacterial communities and inhibited microbial respiration regardless of the N fertilizer application rates (**Chapter 5**).

- Significant correlation between nitrifying and denitrifying communities and their activities were observed (**Chapters 2, 3, and 4**). This provided novel insights into the relationship "microbial communities-ecosystem functioning" which is still not fully known. In other words, soil microbial communities may be not functionally redundant, and thus impacts of environmental changes on soil functional communities may significantly alter the rate of functions.
- AOA and AOB co-habited irrigated cotton soils, and AOA outnumbered their counterparts AOB (**Chapters 2, 3, and 4**). There were no clear niche differentiations of AOB and AOA in response to waterlogging stress and combined elevated CO_2 and temperature (**Chapters 2 and 3**) whereas only AOA responded to elevated temperature (**Chapter 3**) and only AOB responded to drought legacy effects and N fertilizer addition (**Chapter 4**). It suggested that AOB was more vulnerable to desiccation and more strongly responsive to N supply than AOA in irrigated cotton soils. Additionally, AOB may take a long time to recover after exposed to extreme drought events in irrigated cotton soils (**Chapter 4**).

Overall, extreme weather events and climate change significantly affected soil biotic and abiotic properties, leading to changes in the soil nutrient level which was linked to cotton crop productivity. Waterlogging stress created unfavorable conditions such as increased soil moisture content, thereby potentially leading to hypoxia or even anoxia which negatively affected nitrifying communities. Shifts in ammonia-oxidizing community structure and decreased abundance of AOB and AOA due to waterlogging resulted in a decrease in nitrification rate, and hence reduced soil NO_3^- concentration as a major N source for cotton crop assimilation. The depletion of soil NO_3^- availability might also result from enhanced N loss via leaching and denitrification upon waterlogging (**Figure 6.1**).

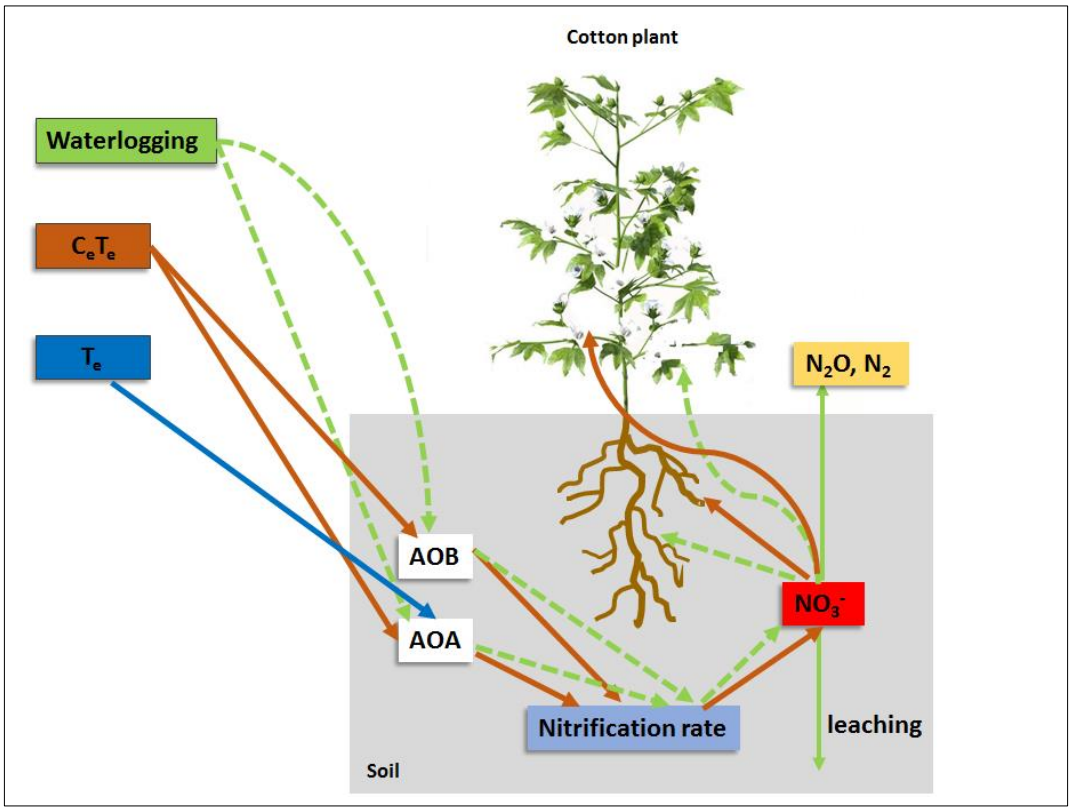

Figure 6.1 Summary diagram showing main effects of waterlogging, elevated CO_2 and elevated temperature on soil, ammonia-oxidizing communities, nitrification rate, soil inorganic N and consequences for crop productivity. Waterlogging decreased the abundance of AOB and AOA and changed ammonia-oxidizing community structure. Elevated CO_2 significantly increased the abundance of AOB and AOA, and changed ammonia-oxidizing community structure. Elevated temperature only significantly affected AOA abundance and structure. Shifts in ammonia-oxidizing communities due to waterlogging and elevated CO_2 led to changes in nitrification activities and soil NO_3^- availability which were linked to crop productivity. Waterlogging might increase N loss through gas emission and leaching. Continuous and dashed arrows indicate positive and negative effects, respectively. Green, brown, and blue arrows denote waterlogging, elevated CO_2, and temperature effects, respectively. Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought. AOB = Ammonia-oxidizing bacteria, AOA = Ammonia-

oxidizing archaea. NO_3^- = Nitrate. CO_2 = Carbon dioxide, N_2O = Nitrous oxide, N_2 = Nitrogen gas. T_e = Elevated temperature, C_eT_e = Elevated CO_2 and temperature.

Elevated temperature (+1.1^oC) did not impact soil physicochemical properties and ammonia-oxidizers whereas elevated CO_2 in combination with elevated temperature significantly affected soil physicochemical properties and ammonia-oxidizing community (**Figure 6.1**). The AOA community responded to elevated temperature (T_e) at the early flowering stage of cotton plants; however, the overall nitrification kinetics did not change, possibly due to shifts in the dormant fraction of AOA. The increased abundance of ammonia-oxidizers and shifts in AOB and AOA community structure in response to combined elevated CO_2 and temperature (C_eT_e) resulted in higher nitrification rates. The response of AOB and AOA to C_eT_e were only apparent when cotton plants reached the early flowering stage. It suggests that the response of soil nitrification and ammonia-oxidizers to future climatic conditions depends on cotton crop growth stage. However, the impacts of elevated temperature and CO_2 on these variables might be obscure due to short experimental duration (approximately 3 months).

Extreme weather events, waterlogging and prolonged-drought prior to planting, established legacy effects on soil biotic and abiotic properties and subsequent consequences on crop productivity (**Figure 6.2**). In particular, soil exposed to waterlogging events had legacy effects on denitrifying community and soil NO_3^- concentration in the following season. However, N fertilizer application compensated the legacy effects of waterlogging on soil NO_3^- content whereas the application of N fertilizer increased the abundance of denitrifiers and altered denitrifying community structure. N fertilizer addition increased N mineralization and nitrification rates, thereby increasing plant-available N contents in the soils. Consequently, legacy effects of waterlogging established at pre-sowing was counteracted and thus crop productivity was maintained. An increase in N mineralization rate after N fertilizer addition could be attributed to increases in readily mineralizable soil organic N and microbial activities.

The abundance of AOB and community structure were strongly correlated with their function. Therefore, N supply increased AOB abundance and altered community structure, resulting in an increase in the rate of nitrification. Similarly, a strong correlation between the abundance of denitrifying community and denitrification activity was observed in my study. Despite a higher abundance of denitrifiers due to waterlogging prior planting, soil was well-aerated during the experiment and hence crop productivity was maintained. However, if waterlogging occurs again, soil N loss via gas emission may be enhanced due to high abundance of denitrifiers, and thus negatively affecting crop productivity.

Soil exposed to prolonged-drought placed strong legacy effects on soil nutrient levels, soil processes, AOB and denitrifying communities (**Figure 6.2**). Although N fertilizer application improved soil nutrient availability through increases in N mineralization and nitrification rates, the negative legacy effects of prolonged-drought on soil properties and crop productivity could not be removed completely. AOB was stimulated by N fertilizer, particularly increased AOB abundance and shifts in AOB community structure; however, they did not recover completely, resulting in lower N mineralization and nitrification rates in prolonged drought soils. The duration of the experiment was approximately 6 months, suggesting that soil microbes may take a longer time to recover from the legacy impact of drought. Therefore, crop productivity decreased due to the depletion of microbial activities and soil nutrition resulted from prolonged-drought event prior to planting.

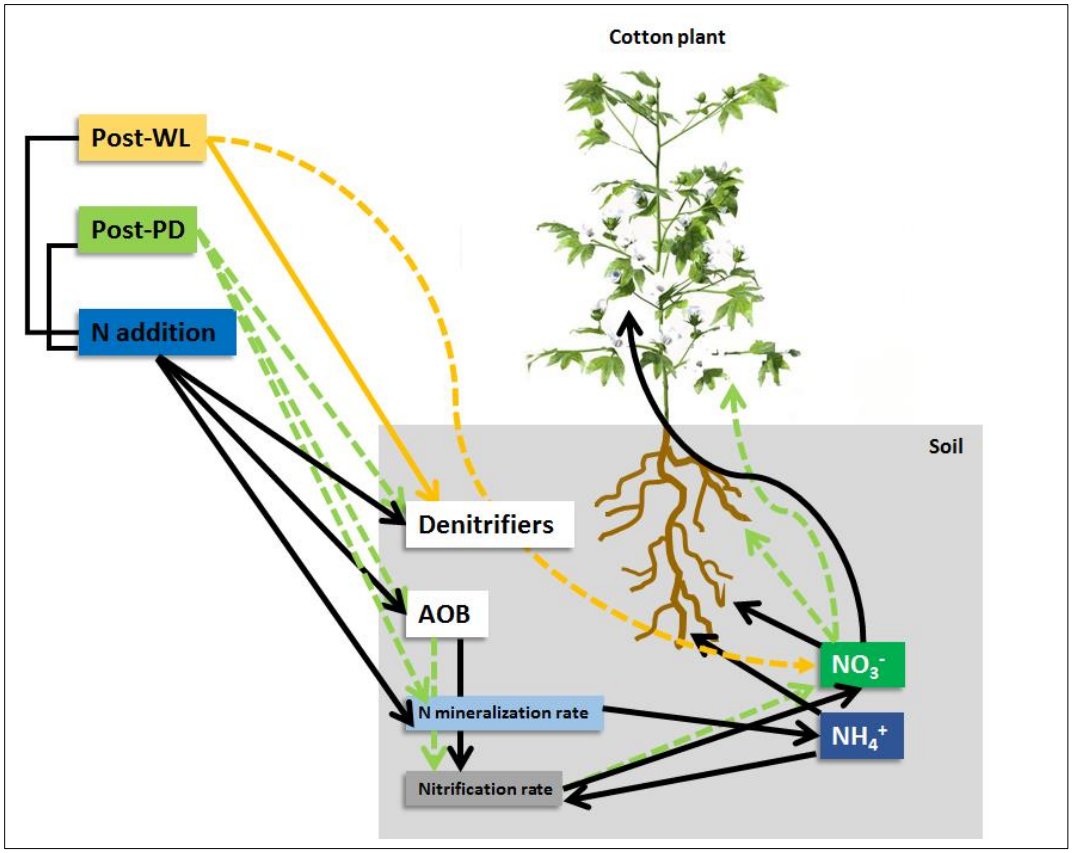

Figure 6.2 Summary diagram showing legacy effects of Post-WL and Post-PD, and the interactive effects of N addition and these legacy effects on soil processes and microbial communities, and consequences for crop productivity. Prolonged-drought event prior to planting established strong legacy effects on AOB and denitrifying communities, nitrification, and N mineralization. Waterlogging prior to planting established legacy effects only on denitrifying community and soil NO_3^- level. N fertilizer addition stimulated AOB and denitrifying community abundance and altered community structure of these microbes. The interaction of N fertilizer addition and drought legacy effects significantly influenced AOB and denitrifying communities. The interaction of N fertilizer addition and waterlogging legacy effects significantly affected denitrifying community abundance and structure. Changes in nitrification and mineralization strongly affected inorganic N pools which were related to crop productivity. Continuous and dashed arrows indicate positive

and negative effects, respectively. Yellow and green arrows denote the legacy effects of drought and waterlogging, respectively. Black arrows denote N fertilizer addition effects, and the interactive effects of N addition and drought and waterlogging effects, respectively. N addition = Nitrogen fertilizer addition, Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought. NO_3^- = Nitrate, NH_4^+ = Ammonium. AOB = Ammonia-oxidizing bacteria.

Prolonged-drought prior to planting also established a strong legacy effect on soil total bacterial community and C cycling, particularly microbial respiration (**Figure 6.3**). Soil total bacterial abundance and diversity were enhanced by N fertilizer addition. The bacterial community composition was also altered by N supply. However, N fertilizer application up to 300 kg N/ha could not remove completely legacy effects of prolonged-drought before sowing on soil total bacterial community. In terms of microbial respiration, basal and substrate-induced respiration rates were lower in soil exposed to prolonged-drought at pre-sowing. N fertilizer application inhibited soil microbial respiration possibly by inhibiting C-degraded enzyme activities and shifts in the microbial community composition. N fertilizer addition in my study altered total bacterial community composition; however, no or weak correlations between soil total bacterial community composition and microbial respiration rates were observed. Hence, my results support the premise that N supply inhibited soil microbial respiration due to inhibited enzyme activities. My study found the interaction of prolonged drought legacy effects and N fertilizer addition weakened the correlation between soil total bacterial community and microbial respiration. The decreased microbial respiration due to N supply led to an increase in C sequestration, thereby improving the soil nutrient profile which was linked to crop productivity.

Ammonia-oxidizing microbes including AOB and AOA co-exist in irrigated cotton soils and AOA outnumbered their counterpart AOB. Although both AOB and AOA was significantly correlated with the rate of nitrification in my study, AOB exhibited a stronger

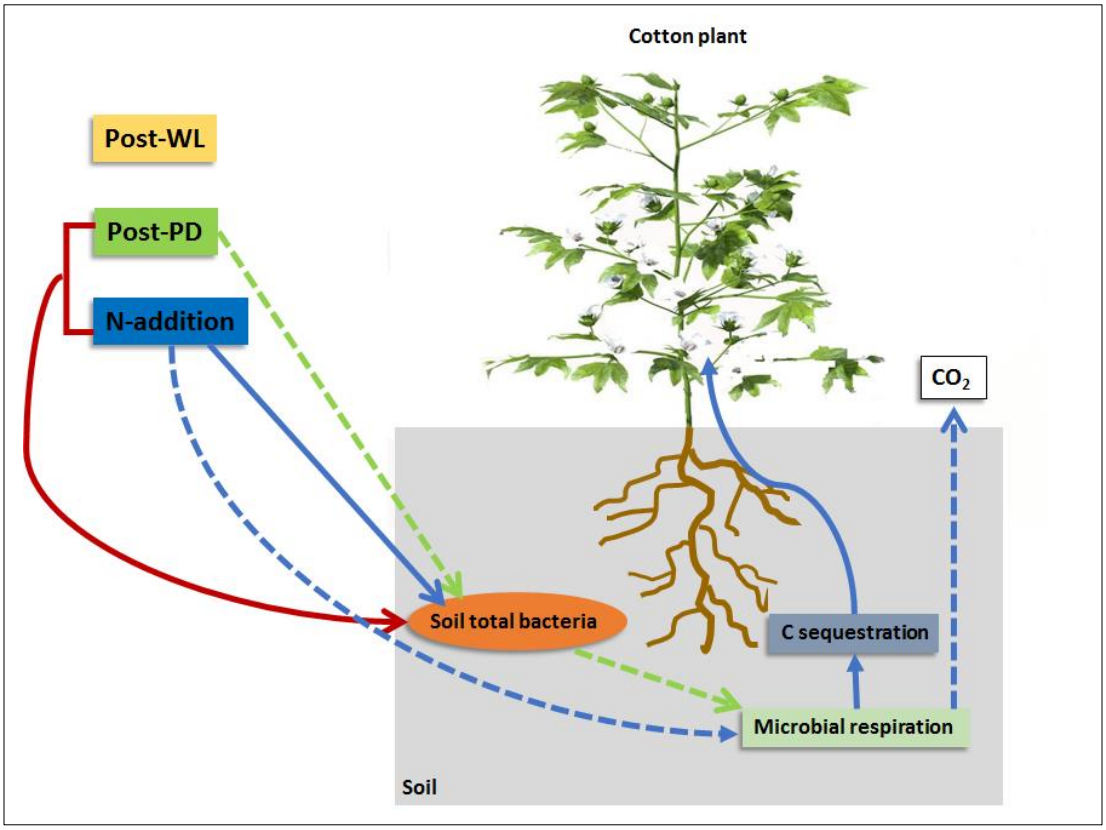

Figure 6.3 Summary diagram indicating legacy effects of Post-PD and the interaction of Post-PD and N fertilizer addition on soil bacterial community and microbial respiration. Post-WL had no legacy effects on soil total bacteria and microbial respiration. In contrast, Post-PD established a legacy effect on soil total bacteria and microbial respiration. N fertilizer addition increased the abundance and diversity of total bacteria, and shifts the total bacterial community composition. N fertilizer addition inhibited soil microbial respiration and hence decreasing C loss from the soil. The interaction of Post-PD and N fertilizer addition altered total bacterial community composition. Inhibited microbial respiration due to N supply potentially led to an increase in soil C sequestration which stimulated crop productivity. Continuous and dashed arrows indicate positive and negative effects, respectively. Green, blue, and red arrows denote legacy effects of Post-PD, N fertilizer addition effects and interactive effects of Post-PD and N fertilizer

addition, respectively. N addition = Nitrogen fertilizer addition, Post-WL = Post-waterlogging, Post-PD = Post-prolonged drought, CO_2 = Carbon dioxide, C = Carbon.

correlation with the nitrification activity. It suggests that in agricultural soils where water and N fertilizer is often well-optimized, AOB become more dominantly functional than AOA. The evidence of strong correlations between nitrification rates and ammonia-oxidizing communities in this thesis contribute to elucidate the role of functional microbial community in maintaining their activity under environmental disturbances.

Future perspectives

Although this study employed a polyphasic approach to integrate soil physicochemical properties, soil N process rates and molecular manipulations in elucidation of soil N cycling response to extreme weather events and climate change and subsequent consequences for crop productivity, it excluded differentiation between the functionally active and inactive microorganisms. The identification of functionally active microbes in soil processes, will contribute to generate a comprehensive understanding of the mechanisms explaining the shifts in the process rates.

By exploiting stable isotope probing (DNA-SIP) techniques, the relative contribution of AOB and AOA to nitrification will be elucidated, thereby fully comprehending and quantifying nitrification response to environmental changes. Additionally, the “meta-omic” techniques including metagenomics, metatranscriptomics, and metaproteomics are a promising approach to identify potential capability and underlying mechanisms involved in controlling the N process rates.

Importantly, to fully assess soil N cycling response to environmental disturbances, the plant properties needs to be included as they indirectly influence the belowground environment. In particular, plant root development should be considered since root exudation may change soil physicochemical properties and microbial communities, thereby potentially influencing N processes in the soils.

For the response of soil nitrification to future climate conditions in Chapter 3, there was lack of full factorial design (i.e no elevated CO_2 treatment alone). Additionally, temperatures inside chambers were lower than expected and the experiment ended when cotton had reached the early flowering stage due to a drop in air temperature in April, 2015. Thus, future works should include the improvement of the experimental design and the whole season experimental operation. Regarding Chapters 4 and 5, prolonged-drought event prior to planting established strong legacy effects on soil and crop productivity; however, the duration of complete resilience of soil microbes has not been determined yet. Therefore, it is important to include this aspect in the future work and run the experiment for a longer time.

Generally, further studies should include above mentioned approaches to provide a complete picture of the responses of soil N cycling to extreme weather events and climate change and subsequent consequences for crop productivity. Such data will be of immense help to develop robust predictive models and adaptation strategies to minimize the impacts of climate change and extreme weather events on agricultural production via effective N management strategies.

APPENDIX A

Table A.1 Two-way ANOVA for the effects of N-addition, soil water treatment legacy, and their interaction on soil physicochemical properties, soil processes and functional microbial communities when cotton plants were at the early squaring stage. Values in bold represent significant differences (P<0.05).

	N-addition		**Soil water treatment legacy**		**N-addition x Soil water treatment legacy**	
	Post-WL	**Post-PD**	**Post-WL**	**Post-PD**	**Post-WL**	**Post-PD**
Soil moisture	0.081	0.375	0.084	0.407	0.865	0.049
pH	**<0.001**	**<0.001**	0.342	0.37	0.185	**0.022**
NH_4^+	**<0.001**	**<0.001**	0.093	**<0.001**	0.896	**0.036**
NO_3^-	**<0.001**	**<0.001**	**0.005**	**<0.001**	0.907	**<0.001**
Total C	0.075	0.081	0.061	**<0.001**	0.401	**0.004**
Total N	**<0.001**	**<0.001**	0.295	**<0.001**	0.917	0.970
PNR	**<0.001**	**<0.001**	0.226	**<0.001**	0.964	0.120
PDR	**<0.001**	**<0.001**	**0.004**	**<0.001**	**0.046**	**0.018**
AOB *amoA* gene abundance	**<0.001**	**<0.001**	0.685	**<0.001**	0.960	0.071
AOA *amoA* gene abundance	0.442	0.546	0.832	0.983	0.886	0.934
nosZ gene abundance	**<0.001**	**<0.001**	**0.004**	**<0.001**	**0.046**	**0.018**

Table A.2 Two-way ANOVA for the effects of N-addition, soil water treatment legacy, and their interaction on soil physicochemical properties, soil processes and functional microbial communities when cotton plants were at the early flowering stage. Values in bold represent significant differences (P<0.05).

	N-addition		**Soil water treatment legacy**		**N-addition x Soil water treatment legacy**	
	Post-WL	**Post-PD**	**Post-WL**	**Post-PD**	**Post-WL**	**Post-PD**
Soil moisture	0.290	0.438	0.399	0.908	0.157	0.127
pH	**<0.001**	**<0.001**	0.455	0.094	0.082	0.537
NH_4^+	**<0.001**	**<0.001**	0.274	**<0.001**	0.979	0.135
NO_3^-	**<0.001**	**<0.001**	0.057	**<0.001**	0.825	**<0.001**
Total C	**<0.001**	**<0.001**	0.718	**<0.001**	0.994	**0.021**
Total N	**<0.001**	**<0.001**	0.076	**<0.001**	0.900	0.391
PNR	**<0.001**	**<0.001**	0.563	**<0.001**	0.996	0.906
PDR	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.109
AOB *amoA* gen abundance	**<0.001**	**<0.001**	0.252	**<0.001**	0.854	**<0.001**
AOA *amoA* gene abundance	0.458	0.292	0.867	0.852	0.999	0.975
nosZ gene abundance	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.109

Table A.3 Two-way ANOVA for the effects of N-addition, soil water treatment legacy, and their interaction on soil physicochemical properties, soil processes and functional microbial communities when cotton plants were at harvest time. Values in bold represent significant differences (P<0.05).

	N-addition		**Soil water treatment legacy**		**N-addition x Soil water treatment legacy**	
	Post-WL	**Post-PD**	**Post-WL**	**Post-PD**	**Post-WL**	**Post-PD**
Soil moisture	0.947	0.506	0.731	0.531	0.934	0.536
pH	**<0.001**	**<0.001**	0.455	0.064	0.082	0.537
NH_4^+	**<0.001**	**<0.001**	0.034	**<0.001**	0.854	**0.020**
NO_3^-	**<0.001**	**<0.001**	0.998	**<0.001**	0.578	**<0.001**
Total C	0.053	**0.032**	**0.021**	**0.013**	0.087	0.064
Total N	**<0.001**	**<0.001**	0.133	**<0.001**	0.800	**0.004**
PNR	**<0.001**	**<0.001**	0.759	**<0.001**	0.884	0.840
PDR	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.385	**0.016**
NNR	**0.001**	**0.003**	0.074	**0.005**	0.076	0.215
MMR	**<0.001**	**<0.001**	0.142	**<0.001**	0.102	0.095
AOB *amoA* gen abundance	**<0.001**	**<0.001**	0.378	**<0.001**	0.938	0.076
AOA *amoA* gene abundance	0.914	0.758	0.582	0.748	0.985	0.999
nosZ gene abundance	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.385	**0.016**

APPENDIX B

Table B.1 Two-way ANOVA for the effects of N-addition, soil water treatment legacy, and their interaction on total bacterial abundance when cotton plants were at the early squaring, flowering stages, and harvest time; and microbial respiration (basal, glucose and lignin-induced respirations) when cotton plants were at the early flowering stage. Values in bold represent significant differences (P<0.05).

	N-addition		**Soil water treatment legacy**		**N-addition x Soil water treatment legacy**	
	Post-WL	**Post-PD**	**Post-WL**	**Post-WL**	**Post-PD**	**Post-WL**
	Early squaring stage					
Total bacterial abundance	**0.005**	**0.006**	0.339	**<0.001**	0.995	0.924
	Early flowering stage					
Total bacterial abundance	**0.001**	**0.001**	0.199	**<0.001**	0.977	0.788
Basal respiration	**0.003**	**0.003**	0.963	**<0.001**	0.886	0.986
Glucose-induced respiration	**0.005**	**0.002**	0.935	**<0.001**	0.997	0.959
Lignin-induced respiration	**<0.001**	**<0.001**	0.235	**<0.001**	0.955	0.555
	Harvest					
Total bacterial abundance	**0.001**	**0.001**	0.571	**<0.001**	0.996	0.978

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